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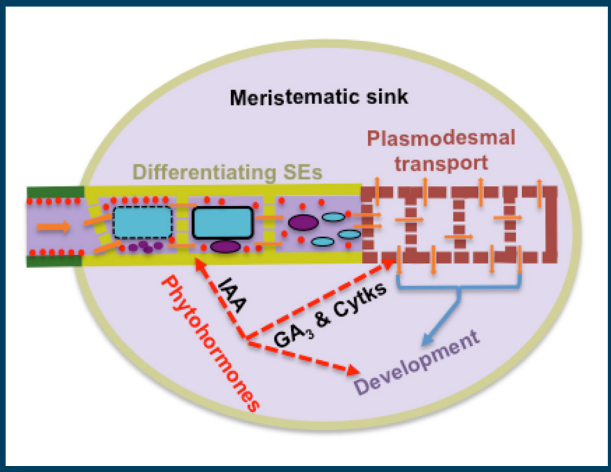

UPTAKE AND REGULATION OF RESOURCE ALLOCATION FOR OPTIMAL PERFORMANCE AND ADAPTATION

Topic Editors
Yong-Ling Ruan, John W. Patrick,
Sergey Shabala and Thomas L. Slewinski

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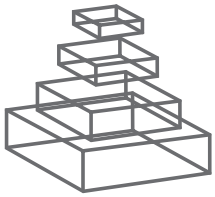

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UPTAKE AND REGULATION OF RESOURCE ALLOCATION FOR OPTIMAL PERFORMANCE AND ADAPTATION

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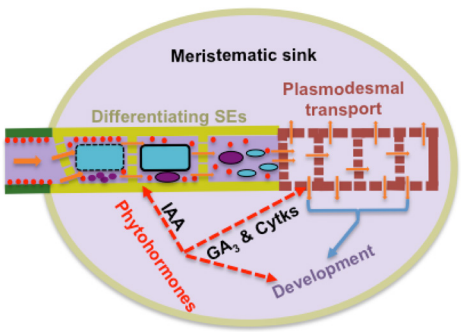

Model of a high resistance pathway encountered by resource flow from transport phloem (green) into meristematic sinks through protophloem SEs differentiating from provascular cells (khaki) arranged in series with symplasmic movement through plasmodesmata of meristematic cells (brown).
Taken from Patrick JW (2013) Does Don Fisher's high-pressure manifold model account for phloem transport and resource partitioning? *Front. Plant Sci.* 4:184.
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Resource allocation is fundamental to plant development, yield formation and tolerance to abiotic and biotic stress. Resource functions in plants in organic and inorganic forms. The former includes organic carbon (C) and nitrogen (N); whereas the latter covers mineral ions and water. In most plants, organic C is initially produced in photosynthetic leaves as sucrose that is transported through phloem to non-photosynthetic tissues (sinks) for diverse uses. On the other hand, ions and water are taken up by roots from soil and transported to the aerial parts through xylem. Organic N is mainly assimilated as amino acids in leaves from organic C and inorganic N for transport through phloem to the remaining plant bodies. The distribution and utilization of resource are overall controlled by concerted actions of membrane-transport proteins and /or metabolic enzymes of cell types located in strategic positions within the plant body. These processes determine plant food, fibre and biofuel quality and yield, hence human and animal nutrition and health and textile and bioenergy production. Importantly,

resources such as glucose, peptides and many ions also act as signalling molecules regulating plant growth and in response to adverse conditions including drought, salt and pathogen infections. Indeed, the dual role of these resource species in nutrient distribution and signalling renders them perhaps ideal molecules in efficiently adjusting plants to adopt some stress situations such as lacking nutrient availability during reproductive stage. As for water, it is estimated that more than 50% of water transported through plant cells are mediated by membrane proteins and increase in water use efficiency has become an urgent matter in global agriculture which is the number one 'consumer' of world water resource.

It has taken humanity thousands of years to create plant varieties that allocate resource into readily harvestable tissues that are compatible with our high intensity agricultural systems. However, most of the changes in resource partitioning conflict with the normal evolutionary trajectories of the particular species. Thus, stabilizing and further modifying resource allocation in plants remains challenging. Given this and in light of the demand to double world crop yield by 2050 to feed more people with less arable land and the increased incidents of climate change-associated abiotic stress such as drought, salt and heat stress, it is imperative to advance our fundamental understanding of the mechanisms underpinning resource uptake and allocation in plants from molecular to whole plant levels. This could allow us to develop innovative solutions to optimise resource distribution and utilization for higher plant yield, quality and tolerance to biotic and abiotic stresses.

Table of Contents

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EDITORIAL
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Uptake and regulation of resource allocation for optimal plant performance and adaptation to stress

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Resource allocation is fundamental to plant development, yield formation and tolerance to abiotic and biotic stress. Resources comprise both organic and inorganic forms. The former includes organic carbon (C) such as sugars; whereas the latter covers mineral ions and water. In most plants, organic C is initially produced in photosynthetic leaves as sucrose that is transported through phloem to non-photosynthetic tissues (sinks) for diverse uses. On the other hand, ions and water are taken up by roots from soil and transported to the aerial parts through xylem. It has taken mankind thousands of years to breed plant varieties that allocate resources into readily harvestable organs that are compatible with high intensity agricultural systems. However, most of the changes in the resource partitioning, imposed by plant breeding, conflict with the evolutionary trajectories of the particular species. Thus, further modifying resource allocation in plants remains challenging. At the same time, food production will need to increase by up to 70% by 2050 to match population growth (e.g., Godfray et al., 2010). Achieving this goal has become increasingly challenging. For instance, availability of arable land is progressively decreasing as a result of urbanization. This is further aggravated by global climate change driving increased frequency and severity of drought, flooding and heat stress events in many regions of the world (Gilbert, 2009; Godfray et al., 2010). Thus, it is imperative to advance our fundamental understanding of the mechanisms underpinning resource uptake and allocation in plants from molecular to whole plant levels. With this background, we believe it is timely to launch this research topic on uptake and regulation of resource allocation. The 14 papers published in the topic highlight some major advances and future directions in the area.

Improving photosynthetic efficiency is a key strategy to increase plant biomass production. In this context, Slewinski (2013) discussed novel ways to engineer kranz-type C4 photosynthesis based on a new hypothesis that describes how the syndrome can repeatedly and rapidly evolve from conserved developmental programs found in C3 angiosperms. Membrane transport between intracellular compartments and cell types play significant roles in mediating flows of photosynthetic products (photoassimilates) from chloroplasts of source leaves to sites of utilization within sinks. To this end, Ludewig and Flügge (2013) provide an insightful update on the expanding array of discovered membrane transporters that contribute to these photoassimilate flows. Apart from efflux to cytosol for synthesis of sucrose, some fixed C in chloroplasts is also used for starch synthesis at daytime that is degraded into glucose and maltose for sucrose synthesis and export at night. Here, Hirose et al. (2013) identified a rice α-glucan water dikinase as a key enzyme for starch degradation, a mutation of which caused hyper-accumulation of starch in source leaves without detectable effects on vegetative growth whilst significantly impeding reproductive development. Rapid homeostatic adjustments of source to sink photoassimilate flows likely depend upon post-translational control of transporter activities. Krügel and Kuehn (2013) review this emerging area of exciting research endeavor with a focus on protein-protein interactions regulating activities of sucrose/H^+symporters (SUT). Mapping SUT expression profiles throughout the source-path-sink system has provided an information base for Milne et al. (2013) to propose potential roles that SUTs may play in accumulating sucrose to high concentrations in Sorghum stems. Understanding mechanisms responsible for determining partitioning of photoassimilates (and other resources) between competing sinks continues to remain elusive. Against the current knowledge of phloem transport biology, Patrick (2013) re-evaluates Don Fisher's high-pressure manifold hypothesis in which plasmodesmata located in the sink phloem are conceived to exert a primary control over photoassimilate partitioning patterns.

Upon reaching sinks, assimilates are unloaded from phloem for sink development and yield formation. To this end, Bihmidine et al. (2013) provide an update on the major molecular drivers of sink strength covering the potential roles of recently identified SWEET sugar uniporters and novel aspects of a sucrose-degrading enzyme, invertase (INV). On a related note, new pathways mediated by INV during maize endosperm development have been explored by Silva-Sanchez et al. (2013) by comparing protein profiles between the INV-mutant (minature1) and its wild-type. Carbon derived from photosynthetically-generated sucrose is used for the synthesis of a diverse range of storage products including cellulose (e.g., Brill et al., 2011), starch (e.g., Ruan et al., 2008) and fructan. Here, Van den Ende (2013) propose two emerging roles for fructans and raffinose family oligosaccharides (RFOs): (i) their contribution to overall cellular reactive oxygen species (ROS) homeostasis by specific ROS scavenging processes and (ii) their action as phloem-mobile signaling molecules under stress. Apart from their nutrient roles, soluble sugars, including sucrose, glucose and fructose and their derivatives such as trehalose and trehalose-6-phosphate, function as signals regulating

gene expression and cell development. The sugars may function as independent signals or through crosstalk with other signaling pathways as highlighted by Wang and Ruan (2013) on the regulation of cell division and expansion by sugar and auxin signaling.

A plant's ability to assimilate carbon and allocate carbohydrates is critically dependent on potassium availability (Cakmak, 2005). In this volume, Sharma et al. (2013) provide a comprehensive overview of molecular mechanisms behind K+ transport and sequestration in the model plant *Arabidopsis thaliana*, and their regulation under diverse stress conditions. Potassium also is a major inorganic osmolyte responsible for cell turgor maintenance and all types of plant movements, from stomata movement to tropisms and tissue expansion growth (Shabala, 2003). As a result, K+ transport in plants is causally related to flow of water between various tissues and cellular compartments. The latter issue is addressed by Prado and Maurel (2013) in which they evaluate the regulation of leaf hydraulics from molecular to whole plant levels emphasizing the role of aquaporins in this process. They also discuss how the above knowledge could help in optimizing plant performance and its adaptation to extreme conditions over short- and long-time scales.

Resource allocation is also central to plant defense responses to abiotic and biotic stresses. The tug of war between plants and their pathogens for resources is highlighted by Schultz et al. (2013). These authors describe the discovery of how plants, as a major defense strategy, mitigate pathogen attack by altering their patterns of resource allocation. In relation to abiotic stress, Liu et al. (2013) assess current understanding of sugar regulation of fruit and seed responses to heat and drought from which they formulate new directions for improving seed and fruit set, which are key determinants of crop yield potential (Ruan et al., 2012).

Overall, it is becoming increasingly clear that uptake and regulation of resource allocation is a rapidly advancing field, with ever increasing significance for sustainable agriculture. We hope this research topic will shed new light on some major aspects of resource allocation and boost research in this area of critical importance for future food security and environmental sustainability.

REFERENCES

Bihmidine, S., Hunter, C. T., Johns, C. E., Koch, K. E., and Braun, D. M. (2013). Regulation of assimilate import into sink organs: update on molecular drivers of sink strength. *Front. Plant Sci.* 4:177. doi: 10.3389/fpls.2013.00177

Brill, E., van Thournout, M., White,R. G., Llewellyn, D., Campbell, P., Engelen, S., et al. (2011). A novel isoform of sucrose synthase is targeted to the cell wall during secondary cell wall synthesis in cotton fibre. *Plant Physiol.* 157, 40–54. doi: 10.1104/pp.111.178574

Cakmak, I. (2005). The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *J. Plant Nutr. Soil Sci.* 168, 521–530. doi: 10.1002/jpln.200420485

Gilbert, N. (2009). The disappearing nutrient. *Nature* 461, 716–718. doi: 10.1038/461716a

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., et al. (2010). Food security: the challenge of feeding 9 billion people. *Science* 327, 812–818. doi: 10.1126/science.1185383

Hirose,T., Aoki, N., Harada, Y., Okamura, M., Hashida, Y., Ohsugi, R., et al. (2013). Disruption of a rice gene for α-glucan water dikinase, OsGWD1, leads to hyperaccumulation of starch in leaves but exhibits limited effects on growth. *Front. Plant Sci.* 4:147. doi: 10.3389/fpls.2013.00147

Krügel, U., and Kuehn, C. (2013). Post-translational regulation of sucrose transporters by direct protein–protein interactions. *Front. Plant Sci.* 4:237. doi: 10.3389/fpls.2013.00237

Liu, Y.-H., Offler, C. E., and Ruan, Y.-L. (2013). Regulation of fruit and seed response to heat and drought by sugars as nutrients and signals. *Front. Plant Sci.* 4:282. doi: 10.3389/fpls.2013.00282

Ludewig, F., and Flügge, U.-I. (2013). Role of metabolite transporters in source-sink carbon allocation. *Front. Plant Sci.* 4:231. doi: 10.3389/fpls.2013.00231

Milne, R. J., Byrt, C. S., Patrick, J. W., and Grof, C. P. (2013). Are sucrose transporter expression profiles linked with patterns of biomass partitioning in Sorghum phenotypes? *Front. Plant Sci.* 4:223. doi: 10.3389/fpls.2013.00223

Patrick, J. W. (2013). Does Don Fisher's high-pressure manifold model account for phloem transport and resource partitioning? *Front. Plant Sci.* 4:184. doi: 10.3389/fpls.2013.00184

Prado, K., and Maurel, C. (2013). Regulation of leaf hydraulics: from molecular to whole plant levels. *Front. Plant Sci.* 4:255. doi: 10.3389/fpls.2013.00255

Ruan, Y.-L., Llewellyn, D. J., Liu, Q., Xu, S. M., Wu, L. M., Wang, L., et al. (2008). Expression of sucrose synthase in the developing endosperm is essential for early seed development in cotton. *Funct. Plant Biol.* 35, 382–393. doi: 10.1071/FP08017

Ruan, Y.-L., Patrick, J. W., Bouzayen, M., Osorio, S., and Fernie, A. R. (2012). Molecular regulation of seed and fruit set. *Trends Plant Sci.* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Schultz, J. C., Appel, H. M., Ferrieri, A., and Arnold, T. M. (2013). Flexible resource allocation during plant defense responses. *Front. Plant Sci.* 4:324. doi: 10.3389/fpls.2013.00324

Shabala, S. (2003). Regulation of potassium transport in leaves: from molecular to tissue level. *Ann. Bot.* 92, 627–634. doi: 10.1093/aob/mcg191

Sharma, T., Dreyer, I., and Riedelsberger, J. (2013). The role of K+ channels in uptake and redistribution of potassium in the model plant *Arabidopsis thaliana*. *Front. Plant Sci.* 4:224. doi: 10.3389/fpls.2013.00224

Silva-Sanchez, C., Chen, S., Zhu, N., Li, Q.-B., and Chourey, P. S. (2013). Proteomic comparison of basal endosperm in maize miniature1 mutant and its wild-type Mn1. *Front. Plant Sci.* 4:211. doi: 10.3389/fpls.2013.00211

Slewinski, T. L. (2013). Using evolution as a guide to engineer kranz-type c4 photosynthesis. *Front. Plant Sci.* 4:212. doi: 10.3389/fpls.2013.00212

Van den Ende, W. (2013). Multifunctional fructans and raffinose family oligosaccharides. *Front. Plant Sci.* 4:247. doi: 10.3389/fpls.2013.00247

Wang, L., and Ruan, Y.-L. (2013). Regulation of cell division and expansion by sugar and auxin signaling. *Front. Plant Sci.* 4:163. doi: 10.3389/fpls.2013.00163

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Flexible resource allocation during plant defense responses

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Plants are organisms composed of modules connected by xylem and phloem transport streams. Attack by both insects and pathogens elicits sometimes rapid defense responses in the attacked module. We have also known for some time that proteins are often reallocated away from pathogen-infected tissues, while the same infection sites may draw carbohydrates to them. This has been interpreted as a tug of war in which the plant withdraws critical resources to block microbial growth while the microbes attempt to acquire more resources. Sink-source regulated transport among modules of critical resources, particularly carbon and nitrogen, is also altered in response to attack. Insects and jasmonate can increase local sink strength, drawing carbohydrates that support defense production. Shortly after attack, carbohydrates may also be drawn to the root. The rate and direction of movement of photosynthate or signals in phloem in response to attack is subject to constraints that include branching, degree of connection among tissues, distance between sources and sinks, proximity, strength, and number of competing sinks, and phloem loading/unloading regulators. Movement of materials (e.g., amino acids, signals) to or from attack sites in xylem is less well understood but is partly driven by transpiration. The root is an influential sink and may regulate sink-source interactions and transport above and below ground as well as between the plant and the rhizosphere and nearby, connected plants. Research on resource translocation in response to pathogens or herbivores has focused on biochemical mechanisms; whole-plant research is needed to determine which, if any, of these plant behaviors actually influence plant fitness.

Keywords: carbon allocation, herbivory, nitrogen allocation, sequestration, plant defense

All parts of an individual plant are connected through the medium of its resource budget" Janzen (1973).

Janzen's observation was intended to place the physiologically obvious in the context of plants as food for insects. He was making the point that as insects harvest material from one plant module, other modules are likely to feel the impact. We now know that those undamaged modules also supply materials for the production of defenses activated by insect attack and that some of the impact of herbivory on many plant organs involves investment in systemic defense production. How plants manage resource allocation in the face of conflicting demands has long intrigued ecologists and has shaped our thinking about plant evolution.

The view that organisms must make choices among investments from a limited resource budget rests comfortably within a framework familiar to both engineers (as "design constraints") and economists (the "no free lunch" principle). It has a long history in biology as well. Darwin (1872) cited the "law of compensation or balancement of growth" of Saint-Hilaire and Goethe from the early nineteenth century, quoting Goethe:

"The budget of nature is fixed; but she is free to dispose of particular sums by an appropriation that may please her. In order to spend on one side, she is forced to economize on the other side" (Saint-Hilaire, 1818).

If a tradeoff exists between investment in defense and investment in other functions that influence fitness, then natural selection may favor the evolution of inducible defenses, whose expense occurs only when needed (Steppuhn and Baldwin, 2008). The necessary assumption that defense production has fitness costs has mixed support, largely because it is extremely difficult, if not impossible, to manipulate defense without confounding treatment effects. The ability to silence defense production or elicitation using transgenes is undoubtedly the best method we have available for pinpointing potential fitness costs of defense. A cost of producing chemical defenses has indeed been found in the few cases in which this method has been used (Schwachtje and Baldwin, 2008). As the ability to manipulate specific genes in more plant species becomes possible, we hope more such experiments will help clarify defense costs. It is possible that no experimental method will ever isolate cost of defense from other costs or influenced functions, since the gene networks involved in defense intersect with and influence networks regulating other fitness-related functions (Baldwin and Preston, 1999; Steppuhn and Baldwin, 2008). But broadly speaking, immunity to disease or herbivores is thought to incur fitness costs in all organisms, and this provides a justification for the evolution of inducible responses to attack.

RESOURCE ALLOCATION IN DEFENSE

Responses to attack by insects or pathogens create a local need for resources to support defense. But herbivory, wounding and jasmonate decrease *local* photosynthetic rates (Creelman and Mullet,

1997; Beltrano et al., 1998; Hristova and Popova, 2002; Izaguirre et al., 2003; Schwachtje and Baldwin, 2008; Gomez et al., 2010) so that plants cannot add to their carbon pool in response to attack by increasing local carbon fixation. Similarly, evidence that nutrient uptake by roots is stimulated by herbivory is mixed, and uptake is frequently reduced. This means that the building blocks for defense production must be reallocated from existing pools or redirected from current "income" to support defense and provide resistance in response to attack. These materials must be translocated from distant tissues whenever locally available resources are not sufficient to support defense production, as in young, developing leaves (Arnold and Schultz, 2002; Arnold et al., 2004). For example, some have reported increases in photosynthetic activity in unattacked leaves following damage by defoliating herbivores (Nowak and Caldwell, 1984; Welter, 1989; Schwachtje and Baldwin, 2008), and have suggested that this may partially compensate for defoliation (Detling and Painter, 1983; Nowak and Caldwell, 1984). Shoots emerging to replace those lost to browsers, are especially dependent upon resource transport and are typically strong sinks (Iqbal et al., 2012); one result can be apparently increased plant productivity and increased resources for herbivorous insects (Nykänen and Koricheva, 2004). This review discusses how such a transport system might provide plant organs with the materials needed for defense in response to attack by herbivores (and microbes). It focuses on carbon and nitrogen as resources for defense, because those two elements have been the focus of defense theories and differ in how they are acquired and translocated.

The observation that plants comprise modules linked by access to and competition for resources means that resource needs for defense in one module may be met by translocation from other modules, and that two or more demanding modules may compete. Early studies attempted to link plant resource availability to plant defense focused primarily on "local" resources, already available at wound sites. More recently, we have come to understand that plants are a dynamic mosaic of resources and defensive substances. Observed changes in resource flow in response to insect or pathogen attack appear to be complex and sometimes even contradictory. We suggest that altered translocation of resources is a critical part of responses to insect and pathogen attack and needs to be incorporated into thinking about plant defense "strategy."

RESOURCES: CARBON

Plants fix about 560 Gt of atmospheric carbon per year (Jansson et al., 2010) and incorporate it into hundreds of thousands of molecular structures, many of which contain or are derived from sugars. For example, sugars and their direct products represent more than two-thirds of the dry weight of woody plants (Richardson et al., 2013). Plants also amass and store soluble sugars and starch as nonstructural carbohydrates (NSC) to support future respiration and growth as well as metabolic processes generating plant defenses (Chapin et al., 1990). The mass of stored NSCs can also be significant; for example, Würth et al. (2005) estimated that 8% of the living biomass of a tropical forest consisted of soluble sugars and starch. Stored NSCs provide plants with the ability to replace tissues lost to pests and other stresses. For example, temperate trees store enough NSCs to regrow their canopies four times (Millard and Grelet, 2010). Under most circumstances these pools are never fully depleted (see Millard and Grelet, 2010). For some species, cyclitols such as quinic and shikimic acids play a similar role as stored carbon banks (Kozlowski, 1992). While these compounds are the basis of growth and reproduction, many contribute directly or indirectly to the defense of plants against their enemies.

Carbon is also in demand when a plant tissue is called upon to defend itself. This can represent significant metabolic cost in terms of biosynthesis, storage, and eventual fitness impacts (Gershenzon, 1994). Increasing production of many – perhaps all – defensive compounds in response to attack must result in increasing demand for carbon. Carbohydrates support the production of plant phenolics, many of which play a role in defense and can easily exceed >25% of plant dry mass, via the shikimic acid and phenylpropanoid pathways (Harding et al., 2009). Plant terpenes are also carbon-intensive defenses; they are, in general, more expensive to produce per gram than other classes of plant defenses, with additional costs incurred for storage (Gershenzon, 1994). The carbon cost of alkaloids is also high; for instance, nicotine requires 3.62 g of glucose per gram produced, representing about 17% of a tobacco plant's net carbon gain (Wink and Roberts, 1998). Even glucosinolate production can increase the demand for photosynthate by 15% or more (Bekaert et al., 2012). It is clear that ready access to carbon supplies is essential for plant defense as well as for growth.

If the local demand for carbon exceeds the local ability to fix it from CO_2, demand must be met via translocation from other sources. However, sites of carbon assimilation and storage (sources) can be quite distant from tissues where utilization creates demand (sinks), requiring translocation over considerable distances. Vascular connections integrate these sites, facilitating carbon transport from sources to sinks to manage the metabolism, growth, and differentiation of shoots, stems, roots, and reproductive structures (Kramer and Kozlowski, 1979). Sucrose is the main transport sugar in most plants, where it may represent >95% of transported carbon (Thorne and Rainbird, 1983; Kozlowski, 1992). Other species transport carbon primarily as larger oligosaccharides and sugar alcohols such as sorbitol. Reducing sugars are rarely transported, if at all (Dinant et al., 2010). The phloem is the major route of carbon transport, although there is evidence for limited xylem transport of carbohydrates in *Acer*, *Betula*, *Salix*, *Vitis*, and *Populus* (Millard and Grelet, 2010). Phloem sap also carries certain defensive compounds, such as alkaloids, flavonoids, and glucosinolates (Turgeon and Wolf, 2009; Wink and Roberts, 1998), as well as information signals such as proteins, RNA, calcium ions, and pressure and/or electrical waves (De Swaef et al., 2013). These substances move en masse in phloem sieve elements along a concentration gradient at rates of 10 cm to 1 m per h, depending on the mechanisms of loading and unloading carbohydrates to phloem sieve tubes (Kozlowski, 1992).

Whether redirecting current photosynthate from sources or reallocating from storage, patterns of carbon movement are constrained by plant phyllotaxy and the arrangement of vascular connections (Jones et al., 1993; Orians, 2005). Even among directly connected plant tissues transport can be affected by tissue size and

distance, disrupted by damage to the phloem (e.g., as in girdling) and interrupted at branch points, for example in *Populus* (Arnold et al., 2004; Appel et al., 2012a).

CARBON SOURCES

Carbohydrates originate in photosynthetic tissues such as mature leaves or storage organs such as roots and stems. These are called "sources." The usual life history for a leaf is that it begins as a sink, dependent on sources for carbohydrate supply. As it matures it transitions from sink to mixed sink and source, to entirely source (exporting CHO). Source leaves may export some or all of their CHOs to storage as well as to sites actively demanding them. Export from sources – from current photosynthesis or from storage – supports the growth and defense of remote tissues and may also increase the efficiency of photosynthetic processes in source leaves by reducing product inhibition (Millard et al., 2007; Ayre, 2011). As for their own defense, mature leaves have age-related limitations; they may tap into their own photosynthate but do not revert to importing sinks. For example, despite the fact that the wound hormone jasmonic acid rapidly reduces photosynthetic electron transport and gas exchange (Nabity et al., 2013), mature source leaves of poplar trees can sustain some induced phenolic synthesis using what is left of their own carbon gain.

Export requires loading sucrose into phloem sieve elements from symplastic or apoplastic compartments via active transport. Apoplastic loading, involving the pumping of extracellular sucrose into phloem cells, is most common and may include sugar alcohols (Sauer, 2007). This is facilitated by the sucrose transporters (SUT), sucrose carriers (SUC), or more generally the monosaccharide transporters, which are driven by negative plant potentials. Chen et al. (2012) recently showed that apoplastic sucrose loading in *Arabidopsis* is a two-step process that requires the activity of two SWEET sucrose efflux transporters, AtSWEET11 and 12. This type of active phloem loading and phloem transport can be disrupted by loss-of-function mutations or pharmacological inhibitors of these transporters (e.g., Geiger, 2011; Geiger and Savonick, 1975; Turgeon and Gowan, 1990). A second type of active loading occurs symplastically via the polymer trapping mechanism and involves interconnected intermediary cells and the production of raffinose-type oligosaccharides (Fu et al., 2011).

These active loading mechanisms are common in herbaceous plants, which can concentrate sucrose to concentrations as high as 1 M and generate relatively high phloem pressures (Fu et al., 2011; Geiger, 2011). On the other hand, phloem loading also occurs passively by diffusion, sometimes facilitated by membrane proteins called sucrose facilitators (SUF; as in Ayre, 2011), and this is seemingly restricted to woody species exhibiting lower phloem pressures (Gamalei, 1989; Rennie and Turgeon, 2009). It has been proposed that these differences in loading mechanisms, and therefore phloem pressures, among plants may influence patterns of carbon partitioning, defense, and CO_2-responses (Korner et al., 1995; Long et al., 2006; Turgeon, 2010; Fu et al., 2011).

CARBON SINKS

Sinks are tissues to which photosynthate is drawn from sources. Sink strength, a tissue's relative ability to draw in and unload photosynthate, is determined by the activities of enzymes such as sucrose-cleaving invertases and H^+ coupled transporters as well as by proximity to exporting sources and competing sinks. Cell wall invertase (CWI), an enzyme that is ionically bound to plant cell walls, facilitates phloem unloading at sink tissues by cleaving sucrose into fructose and glucose (Sturm and Tang, 1999). Sugar transporters also contribute to carbon unloading at sinks. The activity of sink enzymes is tightly regulated by transcriptional, post-transcriptional, and post-translational mechanisms (Liesche et al., 2011) which are, in turn, affected by mechanical damage, grazing, infection, galling, all classes of plant hormones, and the development of floral organs, fruits, and seeds, among other tissue types (Roitsch and González, 2004).

Young, developing leaves, fruits, and roots are sinks, and do not possess the resources required to support their own growth and development. Because their demand for sugars is unmet by local supply, carbohydrates are imported. Phloem unloading at these sites may be symplastic or apoplastic (Wolswinkel, 1985), with or against a concentration gradient (Fondy and Geiger, 1982). Tissues may be meristematic, elongation, respiratory, or storage sinks depending on their primary "need" (Chen et al., 2001). Leaves, stems, and coarse roots may transition to source tissues capable of exporting carbohydrates as they age, but developing fine roots, fruits, flowers, seeds, or nectories are sinks their entire lives (e.g., Vassilyev, 2010).

COMPETITION AMONG SINKS

Since sink strength is defined as a tissue's ability to import photosynthate relative to other tissues, it is assumed that sink tissues may compete for available carbohydrates. Plant ontogeny is characterized by a predictable sequence of shifting sink priorities, from germination through reproduction. For example, the roots and stems of many tree species alternate sink status to facilitate CHO flow to growing roots in the early season before bud break, then to emerging buds, and back to storage sites in roots and stems in the fall (Shiroya et al., 1966; Ziemer, 1971; Teskey and Hinckley, 1981; Nguyen et al., 1990). Meristems, flowers, fruits, or roots may be the strongest sinks at various times during plant development. Evidence for sink competition can be seen in the impacts of the common horticultural practice of removing competing sinks such as buds, flowers, or fruits (e.g., Smith and Holzapfel, 2009). This redirects CHO flow to remaining sink modules without altering the dry matter production of the plant as a whole (Ho, 1988; Berninger et al., 2000). Herbivores, such as larger browsers, alter CHO flow patterns when they remove source and sink tissues unevenly.

Sinks created by insect or pathogen attack are also potential competitors for photosynthate, both with the plant's normal sinks and each other. Sink-driven competition is best characterized for galls elicited by aphids. Inbar et al. (1995) found competitive interactions among galls of several aphid species on pistachio leaves. Larson and Whitham (1997) demonstrated competition between aphid galls and meristematic sinks on cottonwood trees in which the number of buds drawing resources negatively impacted gall success.

The development of strong, competing sinks is the basis of the negative impacts on wheat and rice growth by the galling pests, Hessian fly and rice gall midge (Harris et al., 2006; Williams et al.,

2011). Sink creation is widespread among galling insect species (Bronner, 1992) and has been shown for free living insect herbivores as well; it should be regarded as an important element of the impact insects can have on their host plants as well as on each other (Baldwin and Preston, 1999; Allison and Schultz, 2005).

Bacterial and fungal pathogens and symbionts also create sinks at the point of infection (Hatcher, 1995; Schaarschmidt et al., 2006). They may compete for limited resources, drawing them away from processes such as grain filling in wheat (Bancal et al., 2012). Microrganisms may also interact with defense responses in more complex ways; for example, foliar wound responses in *Medicago* induce plant-wide increases in CWIs which have a positive effect on mycorrhization (Landgraf et al., 2012). Microrganisms should be factored into attempts to understand resource partitioning when plants response to grazers.

Induced sink strength also can be "cooperative"; for instance, poplar leaves immediately adjacent to induced leaves also exhibit enhanced carbon import and defense responses when sink strength in the neighboring leaf is elicited (Arnold et al., 2004). And galls of the aphid *Hormaphis hamamelidis* (Homoptera: Hormaphididae), which form strong sinks on witch hazel leaves, acquire even more resources when located near fruits, which are also strong sinks (Rehill, 2001).

Some sink-inducing pests appear to have little or no impact on others. For example, reciprocal interactions between root-feeding nematodes and leaf-feeding caterpillars on tobacco was found to be independent of sink strength (Kaplan et al., 2011), and some of the galling aphid species on pistachio leaves do not exhibit negative impacts on each other (Inbar et al., 1995). Browser impacts on plants can create regrowth that is more or less suitable for insect herbivores, depending on the system and time of year (Baldwin and Schmelz, 1994; Nykänen and Koricheva, 2004; Iqbal et al., 2012).

RESOURCES: NITROGEN

Nitrogen is often a growth-limiting nutrient for plants, and is required for the production of all plant defenses against herbivores and pathogens either as a component of biosynthetic enzymes or of the defenses themselves, such as proteinase inhibitors, chitinases, alkaloids, and glucosinolates.

Inorganic nitrogen as nitrate and ammonium is taken up by roots and is incorporated into amino acids (glutamine and glutamate) used to synthesize other amino acids and nitrogenous compounds. This takes place in root or shoot, depending on the molecular species of nitrogen taken up and the carbon/nitrogen balance of the plant (Andrews et al., 1986; Marschner, 1995; Kruse et al., 2002). These synthesized amino acids move to sink organs (e.g., growing roots and leaves, flowers, and seeds) that are largely dependent on long-distance supply of nitrogen (Pate, 1973; Pate et al., 1983) *via* xylem. Xylem-to-phloem and phloem-to-xylem transfer occurs in the root and the stem. Some nitrate is reduced in the leaves and exported to the root and shoot apex via phloem, producing amino acid cycling (Okumoto and Pilot, 2011). Plant species that produce nitrogen-intensive defense compounds like alkaloids or glucosinolates may transport those products over long distances from points of synthesis (often roots) to points of action (e.g., Baldwin et al., 1994; Chen et al., 2001).

Plants may store nitrogen as protein for protracted periods. Vegetative storage proteins can be assembled rapidly and broken down rapidly for transport to N-demanding sinks via phloem (Staswick, 1989). Amino acid nitrogen may be stored for months or years in stems, roots or foliage as vegetative storage proteins or in active proteins such as RuBisCO (Millard and Grelet, 2010). In the spring developing leaves are strong sinks for stored nitrogen; nitrogen reserves stored overwinter in the bark and wood support up to 90% of new leaf growth in trees (Millard, 1989; Welter, 1989; Millard and Proe, 1991; Sagisaka, 1993; Stepien et al., 1994; Frak et al., 2002; Millard and Grelet, 2010). Changes in N allocation occur naturally in response to the seasons and plant phenology (Millard et al., 2001; Masclaux-Daubresse et al., 2010; Millard and Grelet, 2010); the allocation to bark storage proteins occurs in response waning daylength and colder temperatures (see Millard and Grelet, 2010). Stored nitrogen is allocated among tissues and organs on a competitive basis. For example, removing expanding buds of *Betula pubescens* being supplied by stored proteins redirects allocation to remaining buds or other tissues (Lehtilä et al., 2000; Millard and Grelet, 2010). Releasing stored nitrogen also allows plants to cope with stress and herbivore attack, in part by supporting regrowth or compensatory growth and is an important element of a plant's ability to tolerate attack (Millard and Grelet, 2010).

The distribution of nitrogenous compounds *via* xylem transport and xylem-phloem transfers is complexly regulated, and may differ with type of compound, plant species, and function of the nitrogenous compounds (storage, defense, growth). N-demanding tissues appear capable of signaling their need for nitrogen to distant sources (Kohl et al., 2012). Movement of solutes in xylem is related to transpiration rates and may be regulated by hormones such as abscisic acid (ABA) (Desclos et al., 2008). Since smaller, growing leaves have relatively low transpiration rates but a high nitrogen demand, other factors must regulate the flow of nitrogenous compounds to these leaves. Large families of genes encoding amino acid, nitrate, alkaloid, and glucosinolate transporters have been identified (Chen et al., 2001; Fan et al., 2009; Shitan et al., 2009; Nour-Eldin et al., 2012). These transporters are involved in loading their substrates into xylem, unloading at sinks, and transferring substrates between xylem and phloem (Gessler et al., 2003). The activities of many of these transporters have not been characterized, and a coherent, detailed model of the regulation of transport via xylem has not yet emerged.

TYPES OF RESOURCE REALLOCATIONS IN PLANT DEFENSE RESPONSES

WOUND-INDUCED SINKS AND RESOURCE IMPORT

Both herbivore and pathogen attack begin as localized events. Both also suppress photosynthesis (Creelman and Mullet, 1997; Beltrano et al., 1998; Hristova and Popova, 2002; Izaguirre et al., 2003; Kocal et al., 2008; Schwachtje and Baldwin, 2008; Gomez et al., 2010). Therefore, the ability of individual plant tissues to accumulate defensive substances in response to attack depends on a supply of photosynthate, nitrogen-containing compounds, and other building blocks. This demand can be met by increased sink strength at the attack site, drawing materials from distant sources. The strong connection between regulation of source/sink relations and pathogen success makes sink-regulating apoplastic invertases

a key part of plant defense against microbes, too (Berger et al., 2007). It seems clear that long-distance transport of resources is a component of plant defense responses to insects and pathogens (Truernit and Sauer, 1995; Arnold and Schultz, 2002; Roitsch et al., 2003; Roitsch and González, 2004; Matyssek et al., 2005; Appel et al., 2012a,b).

A dramatic increase in local sink strength can be triggered by insect attack, microbial infection, mechanical wounding, natural systemic signals, and artificial elicitors in tomato (Ohyama and Hirai, 1999), carrot (Sturm and Chrispeels, 1990), goosefoot (Ehness et al., 1997), pea (Zhang et al., 1996), hybrid poplar trees (Arnold and Schultz, 2002; Arnold et al., 2004; Philippe et al., 2010; Appel et al., 2012a), and oaks (Allison and Schultz, 2005) among others. Increases in sink strength elicited by infection or herbivory enhance the import of phloem contents to those tissues. For example, insect grazing, jasmonic acid, and mechanical damage increased the import of carbohydrates to developing leaves of *Populus* by as much as 400% and contributed to the synthesis of polyphenols (Arnold and Schultz, 2002; Arnold et al., 2004). When CHO import was disrupted by girdling or the removal of nearby source leaves, the production of these substances was reduced or eliminated. Frost and Hunter (2008) found that red oak seedlings increased allocation of ^{13}C from roots to caterpillar-grazed foliage and reduced transport to fine roots.

Ferrieri et al. (2013) examined the impact of MeJA on ^{11}C flow in *Arabidopsis thaliana* over 2–24 h. In this case, ^{11}CHOs were rapidly exported from labeled source leaves and transported to the MeJA-treated sink leaves where they were incorporated into cinnamic acid, a precursor for phenolic biosynthesis. Export to roots also increased at the same time. Ferrieri et al. (2012) also demonstrated that a radioactive glucose, [^{18}F]fluoro-2-deoxy-D glucose, was transported toward wounded *Arabidopsis* leaves and incorporated into phenolic glycosides. In these experiments, like those in hybrid poplar, increases in invertase activity and the accumulation of phenolic compounds were observed in young leaves. Interestingly, in *Arabidopsis* phloem flow began within 2 h, before sink tissues exhibited measurable induced CWI activities. In addition, the accumulation of phenolics in leaves was reduced in mutants lacking SUT needed for phloem loading at source tissues (Ferrieri et al., 2012), suggesting that the flow of CHOs toward wounded tissues may be regulated in part by sources as well as by sinks.

Not all tissues can respond to the same extent with increased sink strength. The ability to increase sink strength, like other induced defense responses, wanes with tissue age. For example, poplar tree leaves lose their ability to respond consistently to elicitation with increased CWI activity as they age and become sources (Arnold et al., 2004). Both invertase activity and ^{13}C import decrease as poplar branch and leaf lengths increase and the age structure of the leaves on them changes toward older (Appel et al., 2012a). At some point aging source leaves cannot not revert to importing sinks when elicited, although they may slow their rates of CHO export in response to wounding or jasmonate (Nowak and Caldwell, 1984; Welter, 1989; Schwachtje and Baldwin, 2008). The ability of a leaf to respond to elicitation can change dramatically with its sink/source status over a few days. Studies of induced defenses as well as sink strength need to account for the progressive and sometimes rapid transition from sink to source seen in most plant tissues.

These relationships among elicitation, sink strength, and import of materials have been studied mainly in plants whose defense chemistry is dominated by carbohydrates. The impact of sink strength on induced defenses that include significant amounts of nitrogen or other elements is not as clear. Nitrogen transport and partitioning can be altered by treatment with the wound hormone, jasmonic acid (e.g., Beardmore et al., 2000; Rossato et al., 2002; Meuriot et al., 2004). Nicotine, which is 17% nitrogen by mass and requires ∼6% of the nitrogen assimilated by roots, is synthesized in the roots of tobacco and transported to leaves via the xylem stream in response to aboveground jasmonic acid (JA), wounding or herbivory (Baldwin et al., 1994). This movement has not, however, been associated with induced sink strength in elicited leaves.

We would expect that movement of any compounds traveling in phloem would be subject to JA/damage/infection-induced sink strength, but compounds moving in xylem would not. Appel et al. (2012a) found that JA treatment of the young leaves failed to change rates of ^{15}N transport from roots to leaves in year-old poplar saplings. ^{15}N transport from roots also was not disrupted by steam girdling, indicating that the ^{15}N label was largely restricted to xylem and independent of source-to-sink phloem flow (Appel et al., 2012a). This was confirmed in larger, 4-year-old trees, using whole branches, longer transport distances, and multiple sampling intervals (Arnold et al., 2004; Appel et al., 2012b). JA treatment led to significantly lower rates of ^{15}N import at 24, 48, and 72 h. It is possible that nitrogen from sources other than roots might have reached the elicited leaves (e.g., stems; Welter, 1989; Fan et al., 2009); that nitrogen would not be labeled and hence would not be detected. Also, it is not clear in what form the ^{15}N would have been transported. The most common form in poplar would be amino acids, and there is generally little transfer of amino acids from xylem to phloem in that genus (Gessler et al., 2003). The most likely interpretation of these results is that nitrogen acquired by roots is translocated to leaves in a form that is restricted to xylem, at least until it reaches the leaves. On the other hand, the widely-observed increase in alkaloid movement from roots to attacked leaves in the Solanaceae clearly indicates an increase in the loading of nitrogenous compounds into xylem in response to aboveground signals (Baldwin et al., 1994).

Evidence to date suggests that the import of CHOs to wounded plant tissues *via* enhanced sink strength is a common response. However, there is less evidence for sink-driven import of N by wounded tissues in most plants. And as mentioned above, some of the mechanisms likely to determine loading and unloading of nitrogenous compounds for transport may be responsive to stimuli or signals originating at distant (e.g., attacked) locations (Davis et al., 1991). But because many nitrogen- or sulfur-rich molecules travel in xylem, others in phloem, and others still in both/either, it is not possible at this point to develop a general model of the impact of local infection or herbivory on nitrogen transport.

Increased sink strength by definition increases the flow of sugars to the sink site, irrespective of the elicitor or tissue function. Sucrose and glucose are signals, and alter diverse physiological functions, including the synthesis of phenolics

(Rolland et al., 2006). It would appear that any strong sink is likely to accumulate phenolics, irrespective of the tissues's function (Appel et al., 2012b). This is particularly conspicuous when a sink is created, for example by aphids, to draw amino acids or other low-concentration nitrogenous materials, producing an excess of sugars at the sink site. Examples of this phenomenon might include aphid galls (Rehill and Schultz, 2012) and very young, developing leaves, both of which accumulate large amounts of anthocyanins despite an apparent lack of defensive function for these compounds (Gould and Lister, 2005). Similarly, blocking CHO flow often also causes sugars to accumulate at upstream locations, sometimes visible as anthocyanins, a process some have called termed "pseudo-induction" (Arnold et al., 2004; Steele et al., 2006). We suspect that accumulation of phenolics at sinks, especially anthocyanins, and perhaps other sugar-intensive chemicals is not defensive, or if there is a defensive role this is an exaptation following on a need to dispose of excess sugars (Gould and Lewontin, 1979; Rehill and Schultz, 2012; Appel et al., 2012b).

WOUND-INDUCED EXPORT AND PLANT TOLERANCE

Wounding, JA treatment, or insect attack have been observed to cause a brief increase in the movement of CHOs away from elicited sites (Babst et al., 2005; Schwachtje et al., 2006; Newingham et al., 2007; Thorpe et al., 2007; Babst et al., 2008; Kaplan et al., 2008; Gomez et al., 2010). The interpretation of such results has been similar to that described in the plant pathogen literature, namely that the plant places valuable nutrients out of the reach of attacking herbivores or diseases, preserving resources for later growth and allowing the plant to tolerate herbivory. This has been described as "bunkering" or "sequestering" resources (Babst et al., 2005, 2008; Schwachtje et al., 2006; Newingham et al., 2007; Kaplan et al., 2008; Gomez et al., 2010; Orians et al., 2011). These studies have employed short-lived radioisotopes, most often ^{11}C, to track flow within hours after elicitation. For example, Schwachtje et al. (2006) found that in *Nicotiana attenuata*, simulated grazing and the removal of sink leaf tissues both resulted in an increased flow of carbon to root tissues within 5 h. These plants exhibited prolonged flowering and increased seed capsule production later, although remobilization from belowground stores to accomplish this has not been demonstrated. Gomez et al. (2010) observed similar induced CHO export in tomato, showing that ^{11}C export rate from labeled source leaves increased from 27% to as much as 36%, 4 h after MeJA exposure or with the addition of regurgitant from the herbivore *Manduca sexta* (Steinbrenner et al., 2011).

The allocation of ^{11}C from leaves to roots may require a biological elicitor. ^{11}C labeled source leaves normally exhibit a 1:1 apportionment of carbon to shoots vs. roots but following the application of damage + caterpillar regurgitant 75% of the exported ^{11}C traveled toward the roots (Gomez et al., 2012). From principle component analyses of metabolite profiles these authors hypothesized decreased starch levels in the elicited leaf, suggesting that the mobilization of this carbon pool supported respiration and defense processes. Babst et al. (2005) reported a similar response in trees, observing that ^{11}CHOs were exported from mature source leaves of *Populus tremuloides* and *P. nigra* saplings at an increased rate when the ^{11}C-exposed leaf was itself treated with jasmonates. ^{11}C was allocated mainly to lower stems and roots within a few hours (also see Bassman and Dickmann, 1985). However, a subsequent experiment in which jasmonate treatments were replaced by insect herbivory showed that acropetal ^{11}C transport from grazed LPI7 did occur and increased relative to ungrazed controls. (Babst et al., 2008).

The sequestration studies finding movement of materials away from elicited leaves are not directly comparable with the studies finding a net movement of photosynthate to elicited sites. First, the sequestration studies have all used short-lived radioisotopes and measured transport within minutes or a few (<5) hours of elicitation. Most studies of transport to elicited sites measured transport 12 or more hours after elicitation. Second, sequestration studies have elicited and focused on movement of materials from older leaves, which are partly or entirely sources, while the long-term movement studies focused narrowly on very young leaves that were known to be entirely or nearly entirely sinks. Such young leaves are not yet exporting materials, which is why they need to import for defense. Older leaves (e.g., on poplar trees), can be induced to increase phenolic synthesis but they cannot elevate sink strength, so they must use their own photosynthate for defense.

More recently, Ferrieri et al. (2013) found that ^{11}CO2 fixation and ^{11}C-photosynthate export from a labeled source leaf increased to both roots and leaves by 2 h after MeJA treatment of young *Arabidopsis* leaves. By 24 h, resource allocation toward roots returned to control levels, while allocation to the young leaves increased. These movements were associated with altered CWI activities in both leaves and roots, implicating sink-source regulation as a key allocation mechanism. The emerging picture is one of finely-tuned resource reallocation to both belowground and aboveground sinks early, followed by preferential allocation to aboveground sinks later. Interestingly, these allocation responses by leaves were abolished when roots were chilled, suggesting that roots control aboveground allocation decisions *via* long-distance signaling (Ferrieri et al., 2013).

The role of roots and their connection in source-sink relationships and defensive responses may be influenced significantly by the rhizosphere environment and interactions with other plants. Mycorrhizal infection elevates invertase activity and sink strength in roots (Schaarschmidt et al., 2006) and the mycorrhizae provide connections with through which photosynthate and signals are transferred to other plants (Simard, 2009; Song et al., 2010). Presumably the same source-sink interactions that manage translocation within a plant also govern transfers between plants (Simard, 2009). In communities with such mycorrhizal connections the sink strength of nearby plants ought to compete with sinks of their neighbors, with community-level consequences.

Additional resources drawn to root sinks also are exuded into the rhizosphere, where they influence nutrient availability and the composition and function of the microbiome there (Walker et al., 2003; Frost and Hunter, 2008). Marschner (1995) estimated that up to 21% of all photosynthetically fixed carbon is transferred to the rhizosphere this way. One can hypothesize a role for roots in directing traffic among competing sinks within the same plant and among connected plants in a community, a view somewhat convergent on Darwin's (1880) view of roots as the plant's organizing center (or "brain").

Remobilization of nitrogen away from sites of fungal or bacterial infection is commonplace and usually interpreted as an attempt by the plant to deprive the pathogen of necessary nutrients (Pageau et al., 2006; Berger et al., 2007). Whether plants do this in response to insect attack has not received as much attention. Newingham et al. (2007) showed N export away from roots of *Centaurea maculosa* attacked by an insect, *Agapeta zoegana*. Infested plants shifted N flow to shoots, translocating almost twice as much N to the shoot even as root grazing reduced total N uptake by 30–50%. These authors hypothesized that this response helps *C. maculosa* tolerate this herbivore, maintaining biomass and a perhaps even reproductive output, despite the loss of roots. Gomez et al. (2010) found that treatment with the wound hormone MeJA accelerated export of [^{13}N] amino acids from labeled tomato leaves for a brief time after treatment. Increased transport of amino acids glutamate, glutamine, and glycine was described as a ". . .strategy to safeguard valuable resources by storing them in distant tissues away from folivores" (Steinbrenner et al., 2011; see Hanik et al., 2010). Generally, jasmonate, which is produced and accumulates at sites of wounding, herbivory, and other stresses, stimulates nitrogen remobilization by accelerating senescence (Rossato et al., 2002; Desclos et al., 2008).

On the other hand, Millard et al. (2001) and Millett et al. (2005) found no effect of browsing on remobilization of nitrogen away from birch leaves. Grazing often draws nitrogen from increased root uptake or storage to attacked leaves in grasses (Thornton and Millard, 2006, but see Redak and Capinera, 1994). Moreira et al. (2012) found increased nitrogen in phloem apparently traveling toward MeJA-treated foliage (needles) in pine saplings. All of these studies were done on much longer time scales than any of the sequestration studies. It appears that research to date does not permit a clear evaluation of the idea that herbivory induces sequestration or that sink-facilitated import functions for nitrogen resources.

Together, these studies show that some species can transport some resources away from sites of herbivory over a brief period following attack. Removing important nutrients from the reach of immobile plant pathogens, whose rapid growth depends on immediate access to nearby resources, may help limit the attack and protect the plant. But we wonder whether these short-term responses would have any impact on insects, which may feed on a leaf for only a few minutes before moving on and for whom carbon is in excess. Removing nitrogen from a leaf may be more effective, but the evidence for sequestering nitrogen is weaker than for carbon, and mixed for responses to insects compared with responses to pathogens. Resources translocated to roots may be stored there, recycled aboveground, sent to mycorrhizae, exuded into soil, and may even wind up in nearby plants. Until the eventual fate of these resources is determined, it is not possible to know whether they are recovered from roots and used for regrowth or reproduction following attack.

RESOURCES: TO ADVANCE OR WITHDRAW IN THE FACE OF ATTACK?

The evidence suggests that plants employ both strategies in response to wounding. Resource flow can increase, stop, or reverse within hours in response to attack or elicitation (Kleiner et al., 1999; Arnold and Schultz, 2002; Babst et al., 2005; Schwachtje et al., 2006; Newingham et al., 2007; Babst et al., 2008; Frost and Hunter, 2008; Gomez et al., 2010). And the view that moving photosynthate belowground or to sites of attack as evidence of mutually exclusive strategies – to grow or defend, resist or tolerate – is certainly an oversimplification.

The collection of diverse experiments conducting over the past decade reveal a few consistent trends. Attack by both insects and pathogens clearly alters the movement of resources among plant modules. Further, it appears that changes in rate or direction of the movement of photosynthate are under the control of sink-source interactions, probably involving inducible invertases and other phloem loading/unloading systems. Activities of some of these sink-creating systems are regulated by infection and/or herbivory, but more work is needed to identify eliciting mechanisms. The movement of materials traveling in xylem rather than phloem is influenced by transpiration, but other factors that probably direct these movements have not been characterized. Differences in transport stream – phloem vs. xylem – produce differences in resource distribution. Factors like branching, orthostichy, and differential sink strength that can change the course of carbon movement in phloem apparently have little or no impact on xylem-transported materials like peptides and alkaloids. While comparative studies have yet to be done we would expect this to produce more heterogeneity in the distribution of carbon and carbon-dependent defenses than in the distribution of nitrogen and its dependent defenses. This heterogeneity, which is widely observed, may itself provide a kind of resistance to herbivores (Whitham, 1982; Schultz, 1983). Competition among sinks could influence the ability of a plant organ or module to respond effectively to attack. The root is a powerful and influential sink against which all other sinks – irrespective of type or source – must compete, and through which resources for defense may pass before reallocation above ground. We have evidence that the root's influence may extend beyond its sink status, to regulate sink-source interactions aboveground. Travel of materials to many – perhaps all – sinks, including the rhizosphere and other connected plants, passes through the root, which may function as a manifold, directing traffic.

Significant gaps in our knowledge remain, however, and it is increasingly difficult to compare individual studies because of differences in experimental design. Recent studies have employed different herbivores or artificial elicitors, with significant discrepancies in the amount of wounding, the number of treatments, the delay between treatments and observations, and the duration and frequency of those observations. They have also tracked resource flow among different plant modules, of different sink/source status, separated by varying distances, using short or long-lived isotope tracers. Seemingly minor variations in experimental design often generate contrasting results. As a result we are generally unable to predict the directionality of resource flow in all but a few very specific cases.

We presume that resource reallocation is advantageous to plants. However, research on resource translocation in response to attack by pathogens or herbivores has tended to focus narrowly on physiological and biochemical mechanisms; considerably more

work is needed at the whole-plant level to determine which, if any, of these fascinating plant behaviors actually influence plant fitness.

To date no clear, convincing experimental evidence has yet linked induced resource transport to or away from attacked tissues to plant fitness. To characterize any response as defensive, it must preserve or enhance plant fitness. The only induced resource (not defense) translocation response we have discussed here that might actually protect a plant *via* a relatively immediate impact on insect feeding would be short-term increases in carbon or nitrogen import to elicited tissues in support of defense production (e.g., Ferrieri et al., 2012). Changes in defense chemistry fueled by this rapid import could theoretically influence insect behavior and reduce damage to the plant, either directly or indirectly by increasing the effectiveness of predators and parasites (Schultz, 1983). The suppression of insect growth can be a meaningful measure of "defense" provided the impact on the insect can be shown to benefit the plant. For example, it is well established that the export of resources from pathogen infection sites generally preserves plant fitness by inhibiting the spread of the infection. The best-supported case in which source-sink relationship contributes to plant resistance is probably competition between meristem sinks and aphid galls on cottonwood (Larson and Whitham, 1997; Compson et al., 2011). A study indicating that transporting photosynthate to roots preserved and extended reproduction later in *N. attenuata* (Schwachtje et al., 2006) also comes close, but is subject to alternative interpretations. Strictly speaking, assessing the impact of responses on plant fitness can and probably should require at least a two-generation measurement. In general, well-integrated manipulative experiments that change resource movement through time, track the movements of individual molecules over extended periods, and show a net fitness advantage to the plant are needed to reach meaningful conclusions.

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REFERENCES

Allison, S. D., and Schultz, J. C. (2005). Biochemical responses of chestnut oak to a galling cynipid. *J. Chem. Ecol.* 31, 151–166. doi: 10.1007/s10886-005-0981-5

Andrews, M., Mcinroy, S., Sprent, J. I., and Taylor H., (1986). Early growth and final yield of autumn sown *Vicia faba* L cultivars given different forms of fertiliser N over winter. *Plant Soil* 96, 421–427. doi: 10.1007/BF02375147

Appel, H. M., Arnold, T. A., and Schultz, J. C. (2012a). JA responses reconfigure the long-distance of carbon but not nitrogen in poplar. *New Phytol.* 2, 419–426. doi: 10.1111/j.1469-8137.2012.04171.x

Appel, H. M., Arnold, T. A., and Schultz, J. C. (2012b). Is polyphenol induction simply a result of altered carbon and nitrogen accumulation? *Plant Signal. Behav.* 7, 1498–1500. doi: 10.4161/psb.21900

Arnold, T., Appel, H., Patel, V., Stocum, E., Kavalier, A., and Schultz, J., (2004). Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. *New Phytol.* 1, 157–164. doi: 10.1111/j.1469-8137.2004.01157.x

Arnold, T. M., and Schultz, J. C. (2002). Induced sink strength as a prerequisite for tannin biosynthesis in *Populus*. *Oecologia* 2, 585–593. doi: 10.1007/s00442-001-0839-7

Ayre, B. (2011). Membrane-transport systems for sucrose in relation to whole-plant carbon partitioning. *Mol. Plant* 3, 377–394. doi: 10.1093/mp/ssr014

Babst, B. A., Ferrieri, R. A., Gray, D. W., Lerdau, M., Schlyer, D. J., Schueller, M., et al. (2005). Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. *New Phytol.* 167, 63–72. doi: 10.1111/j.1469-8137.2005.01388.x

Babst, B. A., Ferrieri, R. A., Thorpe, M. R., and Orians, C. M. (2008). *Lymantria dispar* herbivory induces rapid changes in carbon transport and partitioning in *Populus nigra*. *Entomol. Exp. Appl.* 128, 117–125. doi: 10.1111/j.1570-7458.2008.00698.x

Baldwin, I. T., Karb, M. J., and Ohnmeiss, T. E. (1994). Allocation of ^{15}N from nitrate to nicotine: production and turnover of a damage-induced mobile defense. *Ecology* 6, 1703–1713. doi: 10.2307/1939630

Baldwin, I. T., and Preston, C. A. (1999). The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208, 137–145. doi: 10.1007/s004250050543

Baldwin, I. T., and Schmelz, E. A. (1994) Constraints on an induced defense: the role of canopy area. *Oecologia* 97, 424–430.

Bancal, M. O., Hansart, A., Sache, I., and Bancal, P., (2012). Modeling fungal sink competitiveness with grains for assimilates in wheat infected by a biotrophic pathogen. *Ann. Bot.* 110, 113–123. doi: 10.1093/aob/mcs094

Bassman, J. H., and Dickmann, D. I. (1985). Effects of defoliation in the developing leaf zone on young Populus × euramericana plants. II. Distribution of ^{14}C-photosynthate after defoliation. *For. Sci.* 31, 358–366.

Beardmore, T., Wetzel, S., and Kalous, M. (2000). Interactions of airborne methyl jasmonate with vegetative storage protein gene and protein accumulation and biomass partitioning in Populus plants. *Can. J. For. Res.* 30, 1106–1113.

Bekaert, M., Edger, P. P., Hudson, C. M., Pires, J. C., and Conant, G. C. (2012). Metabolic and evolutionary costs of herbivory defense: systems biology of glucosinolate synthesis. *New Phytol.* 196, 596–605. doi: 10.1111/j.1469-8137.2012.04302.x

Beltrano, J., Ronco, M., Montaldi, E., and Carbone, A. (1998). Senescence of flag leaves and ears of wheat hastened by methyl jasmonate. *J. Plant Growth Regul.* 17, 53–57. doi: 10.1007/PL00007012

Berger, S., Sinha A. K., and Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. *J. Exp. Bot.* 58, 4019–4026. doi: 10.1093/jxb/erm298

Berninger, F., Nikinmaa, E., Sievänen, R., and Nygren, P. (2000). Modeling of reserve carbohydrate dynamics, regrowth and nodulation in a N-2-fixing tree managed by periodic prunings. *Plant Cell Environ.* 23, 1025–1040. doi: 10.1046/j.1365-3040.2000.00624.x

Bronner, R. (1992). "The role of nutritive cells in the nutrition of cynipids and cecidomyiids," in *Biology of Insect-Induced Galls*, eds J. Shorthouse and O. Rohfritsch (New York: Oxford University Press), 118–140.

Chapin, F. S. III, Schulze, E.-D., and Mooney H. A. (1990). The ecology and economics of storage in plants. *Annu. Rev. Ecol. Syst.* 21, 423–447. doi: 10.1146/annurev.es.21.110190.002231

Chen, L.-Q., Qu, X.-Q., Hou1, B.-H., Sosso1, D., Osorio, S., Fernie, A. R., et al. (2012). Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* 335, 207–211. doi: 10.1126/science.1213351

Chen, S., Petersen, B. L., Olsen, C. E., Schulz, A., and Halkier, B. A. (2001). Long-distance phloem transport of glucosinolates in *Arabidopsis*. *Plant Physiol.* 127, 194–201. doi: 10.1104/pp.127.1.194

Compson, Z. G., Larson, K. C., Zinkgraf, M. S., and Whitham, T. G., (2011). A genetic basis for the manipulation of sink-source relationships by the galling aphid *Pemphigus betae*. *Oecologia* 167, 711–721. doi: 10.1007/s00442-011-2033-x

Creelman, R. A., and Mullet, J. E. (1997). Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 355–381. doi: 10.1146/annurev.arplant.48.1.355

Darwin, C. (1872). *The Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*, 6th Edn. London: John Murray.

Darwin, C. (1880). *The Power of Movement in Plants*. London: John Murray Publishers.

Davis, J. M., Gordon, M. P., and Smit, B. A. (1991). Assimilate movement dictates remote sites of wound-induced gene expression in poplar leaves. *Proc. Natl. Acad. Sci. U.S.A.* 88, 2393–2396. doi: 10.1073/pnas.88.6.2393

Detling, J., and Painter, E. (1983). Defoliation responses of western wheatgrass populations with diverse histories of prairie dog grazing. *Oecologia* 57, 65–71. doi: 10.1007/BF00379563

Desclos, M., Dubousset, L., Etienne, P., Le Caherec, F., Satoh, H., Bonnefoy, J., et al. (2008). A proteomic profiling approach to reveal a novel role of *Brassica napus* drought 22 kD/water-soluble chlorophyll-binding protein in young leaves during nitrogen remobilization induced by stressful conditions. *Plant Physiol.* 147, 1830–1844. doi: 10.1104/pp.108.116905

De Swaef, T., Driever, S. M., Van Meulebroek, L., Vanhaecke, L., Marcelis, L. F., and Steppe, K. (2013). Understanding the effect of carbon status on stem diameter variations. *Ann. Bot.* 111, 31–46. doi: 10.1093/aob/mcs233

Dinant, S., Bonnemain, J. L., Girousse, C., and Kehr, J. (2010). Phloem sap intricacy and interplay with aphid feeding. *C. R. Biol.* 333, 504–515. doi: 10.1016/j.crvi.2010.03.008

Ehness, R., Ecker, M., Godt, D. E., and Roitsch, T. (1997). Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation. *Plant Cell* 9, 1825–1841.

Fan, S. C., Lin, C. S., Hsu, P. K., Lin, S. H., and Tsay, Y. F., (2009). The *Arabidopsis* nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. *Plant Cell* 21, 2750–2761. doi: 10.1105/tpc.109.067603

Ferrieri, A. P., Appel, H., Ferrieri, R. A., and Schultz, J. C. (2012). Novel application of 2-[18F]fluoro-2-deoxy-D-glucose to study plant defenses. *Nucl. Med. Biol.* 39, 1152–1160. doi: 10.1016/j.nucmedbio.2012.06.005

Ferrieri, A. P., Agtuca, B., Appel, H. M., Ferrieri, R. A., and Schultz, J. C. (2013). Temporal changes in allocation and partitioning of new carbon as 11C elicited by simulated herbivory suggest that roots shape aboveground responses in *Arabidopsis*. *Plant Physiol.* 161, 692–704. doi: 10.1104/pp.112.208868

Fondy, B. R., and Geiger, D. R. (1982). Diurnal pattern of translocation and carbohydrate metabolism in source leaves of *Beta vulgaris* L. *Plant Physiol.* 70, 671–676. doi: 10.1104/pp.70.3.671

Frak, E., Millard, P., Le Roux, X., Guillaumie, S., and Wendler, R. (2002). Coupling sap flow velocity and amino acid concentrations as an alternative method to ^{15}N labeling for quantifying nitrogen remobilization by walnut trees. *Plant Physiol.* 130, 1043–1053. doi: 10.1104/pp.002139

Frost, C. J., and Hunter, M. D. (2008). Herbivore-induced shifts in carbon and nitrogen allocation in red oak seedlings. *New Phytol.* 4, 835–845. doi: 10.1111/j.1469-8137.2008.02420.x

Fu, Q., Cheng, L., Guo, Y., and Turgeon, R. (2011). Phloem loading strategies and water relations in trees and herbaceous plants. *Plant Physiol.* 157, 1518–1527. doi: 10.1104/pp.111.184820

Gamalei, Y. (1989). Structure and function of leaf minor veins in trees and herbs: a taxonomic review. *Trees Struct. Funct.* 3, 96–110. doi: 10.1007/BF01021073

Geiger, D. (2011). Plant sucrose transporters from a biophysical point of view. *Mol. Plant* 4, 395–406. doi: 10.1093/mp/ssr029

Geiger, D. R., and Savonick, S. A. (1975). "Effects of temperature, anoxia and other metabolic inhibitors on translocation," in *Transport in Plants I. Phloem Transport. Encyclopedia Plant Physiology*, Vol. 1, eds M. H. Zimmermann and J. A. Milburn (Berlin: Springer-Verlag), 256–286.

Gessler, A., Weber, P., Schneider, S., and Rennenberg, H. (2003) Bidirectional exchange of amino compounds between phloem and xylem during long-distance transport in Norway spruce trees (*Picea abies* [L.] Karst). *J. Exp. Bot.* 54, 1389–1397. doi: 10.1093/jxb/erg146

Gershenzon, J. (1994). "The cost of plant chemical defense against herbivory: a biochemical perspective," in *Insect–Plant Interactions*, ed. E. Bernays (Boca Raton: CRC Press), 105–173.

Gomez, S., Ferrieri, R. A., Schueller, M., and Orians, C. M., (2010). Methyl jasmonate elicits rapid changes in carbon and nitrogen dynamics in tomato. *New Phytol.* 188, 835–844. doi: 10.1111/j.1469-8137.2010.03414.x

Gomez, S., Steinbrenner, A. D., Osorio, S., Schueller, M., Ferrieri, R. A., Fernie, A. R., et al. (2012). From shoots to roots: transport and metabolic changes in tomato after simulated feeding by a specialist lepidopteran. *Entomol. Exp. Appl.* 144, 101–111. doi: 10.1111/j.1570-7458.2012.01268.x

Gould, K. S., and Lister, C. (2005). "Flavonoid functions in plants," in *Flavonoids: Chemistry, Biochemistry and Applications*, eds Oyvind M. Andersen and Kenneth R. Markham (Boca Raton, FL: CRC Press), 397–442. doi: 10.1201/9781420039443.ch8

Gould, S. J., and Lewontin, R. C. (1979). The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proc. R. Soc. B* 205, 581–598. doi: 10.1098/rspb.1979.0086

Hanik, N., Gómez, S., Best, M., Schueller, M., Orians, C. M., and Ferrieri, R. A. (2010). Partitioning of new carbon as and sup1; and sup1;C in *Nicotiana tabacum* reveals insight into methyl jasmonate induced changes in metabolism. *J. Chem. Ecol.* 36, 1058–1067. doi: 10.1007/s10886-010-9835-x

Hatcher, P. E. (1995). Three-way interactions between plant pathogenic fungi, herbivorous insects and their host plants. *Biol. Rev.* 70, 639–694. doi: 10.1111/j.1469-185X.1995.tb01655.x

Harding, S. A., Jarvie, M. M., Lindroth, R. L., and Tsai, C. J. (2009). A comparative analysis of phenylpropanoid metabolism, N utilization, and carbon partitioning in fast- and slow-growing *Populus* hybrid clones. *J. Exp. Bot.* 60, 3443–3452. doi: 10.1093/jxb/erp180

Harris, M. O., Freemanac, T. P., Rohfritschbc, O., Andersonc, K. G., Payneac, S. A., and Moore, J. A. (2006). Virulent hessian fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interactions with wheat. *Ann. Entomol. Soc. Am.* 99, 305–316. doi: 10.1603/0013-8746(2006)099[0305:VHFDCL]2.0.CO;2

Ho, L. C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol.* 39, 355–378. doi: 10.1146/annurev.pp.39.060188.002035

Hristova, V., and Popova, L. (2002). Treatment with methyl jasmonate alleviates the effects of paraquat on photosynthesis in barley plants. *Photosynthetica* 40, 567–574. doi: 10.1023/A:1024356120016

Inbar, M., Eshel, A., and Wool, D. (1995). Interspecific competition among phloem-feeding insects mediated by induced host-plant sinks. *Ecology* 76, 1506–1515. doi: 10.2307/1938152

Iqbal, N., Masood, A., and Khan, N. A. (2012). Analyzing the significance of defoliation in growth, photosynthetic compensation and source-sink relations. *Photosynthetica* 50, 161–170. doi: 10.1007/s11099-012-0029-3

Izaguirre, M. M., Scopel, A. L., Baldwin, I. T., and Ballaré, C. L. (2003). Convergent responses to stress. Solar ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. *Plant Physiol.* 132, 1755–1767. doi: 10.1104/pp.103.024323

Jansson, C., Wullschleger, S. D., Kalluri, U. C., and Tuskan, G. A. (2010). Phytosequestration: carbon biosequestration by plants and the prospects of genetic engineering. *Bioscience* 60, 687–696. doi: 10.1525/bio.2010.60.9.6

Janzen, D. H. (1973). Host plants as Islands. II. competition in evolutionary and contemporary time. *Am. Nat.* 107, 786–790. doi: 10.1086/282876

Jones, C. G., Hopper, R. F., Coleman, J. S., and Krischik, V. A. (1993). Control of systemically induced herbivore resistance by plant vascular architecture. *Oecologia* 93, 452–456. doi: 10.1007/BF00317892

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., and Denno, R. F. (2008). Effects of plant vascular architecture on aboveground–belowground-induced responses to foliar and root herbivores on *Nicotiana tabacum*. *J. Chem. Ecol.* 34, 1349–1359. doi: 10.1007/s10886-008-9541-0

Kaplan, I., Sardanelli, S., Rehill, B. J., and Denno, R. F. (2011). Toward a mechanistic understanding of competition in vascular-feeding herbivores: an empirical test of the sink competition hypothesis. *Oecologia* 166, 627–636. doi: 10.1007/s00442-010-1885-9

Kleiner, O., Kircher, S., Harter, K., and Batschauer, A. (1999). Nuclear localization of the *Arabidopsis* blue light receptor cryptochrome 2. *Plant J.* 19, 289–296. doi: 10.1046/j.1365-313X.1999.00535.x

Kocal, N., Sonnewald, U., and Sonnewald, S. (2008). Cell wall-bound invertase limits sucrose export and is involved in symptom development and inhibition of photosynthesis during compatible interaction between tomato and *Xanthomonas campestris* pv *vesicatoria*. *Plant Physiol.* 148, 1523–1536. doi: 10.1104/pp.108.127977

Kohl, S., Hollmann, J., Blattner, F. R., Radchuk, V., Andersch, F., Steuernagel, B., et al. (2012). A putative role for amino acid permeases in

sink-source communication of barley tissues uncovered by RNA-seq. *BMC Plant Biol.* 12:154–172. doi: 10.1186/1471-2229-12-154

Korner, C. H., Pelaez-Riedl, S., and Van Bel A. J. E. (1995). CO_2 responsiveness of plants: a possible link to phloem loading. *Plant Cell Environ.* 18, 595–600. doi: 10.1111/j.1365-3040.1995.tb00560.x

Kozlowski, T. T. (1992). Carbohydrate sources and sinks in woody plants. *Bot. Rev.* 58, 107–222. doi: 10.1007/BF02858600

Kramer, P. J., and Kozlowski T. T. (1979). *Physiology of Woody Plants.* New York: Academic Press.

Kruse, J., Hetzger, I., Hänsch, R., Mendel, R.-R., Walch-Liu, P., Engels, C., et al. (2002). Elevated pCO_2 favours nitrate reduction in the roots of wild-type tobacco (*Nicotiana tabacum* cv. Gat.) and significantly alters N-metabolism in transformants lacking functional nitrate reductase in the roots. *J. Exp. Bot.* 53, 2351–2367. doi: 10.1093/jxb/erf094

Landgraf, R., Schaarschmidt, S., and Hause, B. (2012). Repeated leaf wounding alters the colonization of *Medicago truncatula* roots by beneficial and pathogenic microorganisms. *Plant Cell Environ.* 35, 1344–1357. doi: 10.1111/j.1365-3040.2012.02495.x

Larson, K. C., and Whitham, T. G. (1997). Competition between gall aphids and natural plant sinks: plant architecture affects resistance to galling. *Oecologia* 109, 575–582. doi: 10.1007/s004420050119

Lehtilä, K., Haukioja, E., Kaitaniemi, P., and Laine, K. A. (2000), Allocation of resources within mountain birch canopy after simulated winter browsing. *Oikos* 90, 160–170. doi: 10.1034/j.1600-0706.2000.900116.x

Liesche, J., Krügel, U., He, H., Chincinska, I., Hackel, A., and Kühn, C. (2011). Sucrose transporter regulation at the transcriptional, post-transcriptional and post-translational level. *J. Plant Physiol.* 168, 1426–1433. doi: 10.1016/j.jplph.2011.02.005

Long, S. P., Ainsworth, E. A., Leakey, A. D., Nösberger, J., and Ort, D. R. (2006). Food for thought: lower-than-expected crop yield stimulation with rising CO_2 concentrations. *Science* 312, 1918–1921. doi: 10.1126/science.1114722

Marschner, H. (1995). *Mineral Nutrition of Higher Plants*, 2nd Edn. London: Academic Press.

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., and Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* 105, 1141–1157. doi: 10.1093/aob/mcq028

Matyssek, R., Schnyder, H., Munch, J.-C., Oßwald, W., Pretzsch, H., and Treutter, D. (2005). Resource allocation in plants – the balance between resource sequestration and retention. *Plant Biol.* 7, 557–559. doi: 10.1055/s-2005-873000

Meuriot, F., Noquet, C., Avice, J. C., Volenec, J. J., Cunningham, S. M., Sors, T. G., et al. (2004). Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. *Physiol. Plant.* 120, 113–123. doi: 10.1111/j.0031-9317.2004.0210.x

Millard, P. (1989). Effect of nitrogen supply on growth and internal nitrogen cycling within deciduous trees. *Ann. For. Sci.* 46, 666–668. doi: 10.1051/forest:19890514

Millard, P., and Grelet, G.-A. (2010). Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiol.* 9, 1083–1095. doi: 10.1093/treephys/tpq042

Millard, P., Hester, A., Wendler, R., and Baille, G. (2001). Interspecific responses of trees depends on sites of winter nitrogen storage. *Funct. Ecol.* 15, 535–543. doi: 10.1046/j.0269-8463.2001.00541.x

Millard, P., and Proe, M. F. (1991). Leaf demography and the seasonal internal cycling of nitrogen in sycamore (*Acer pseudoplatanus* L.) seedlings in relation to nitrogen supply. *New Phytol.* 117, 587–596. doi: 10.1111/j.1469-8137.1991.tb00963.x

Millard, P., Sommerkorn, M., and Grelet, G. A. (2007). Environmental change and carbon limitation in trees: a biochemical, ecophysiological and ecosystem appraisal. *New Phytol.* 175, 11–28. doi: 10.1111/j.1469-8137.2007.02079.x

Millett, J., Millard, P., Hester, A. J., and McDonald, A. J. (2005). Do competition and herbivory alter the internal nitrogen dynamics of birch saplings? *New Phytol.* 168, 413–422. doi: 10.1111/j.1469-8137.2005.01510.x

Moreira, X., Zas, R., and Sampedro, L. (2012). Genetic variation and phenotypic plasticity of nutrient re-allocation and increased fine root production as putative tolerance mechanisms inducible by methyl jasmonate in pine trees. *J. Ecol.* 100, 810–820. doi: 10.1111/j.1365-2745.2011.01938.x

Nabity, P. D., Zavala, J. A., and DeLucia, E. H. (2013). Herbivore induction of jasmonic acid and chemical defences reduce photosynthesis in *Nicotiana attenuata*. *J. Exp. Bot.* 2 64, 685–694. doi: 10.1093/jxb/ers364

Newingham, B. A., Callaway, R. M., and Bassirirad, H. (2007). Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. *Oecologia* 153, 913–920. doi: 10.1007/s00442-007-0791-2

Nguyen, P. V., Dickmann, D. I., Pregitzer, K. S., and Hendrick, R. (1990). Late-season changes in allocation of starch and sugar to shoots, coarse roots, and fine roots in two hybrid poplar clones. *Tree Physiol.* 7, 95–105. doi: 10.1093/treephys/7.1-2-3-4.95

Nour-Eldin, H. H., Andersen, T. G., Burow, M., Madsen, S. R., Jørgensen, M. E., Olsen, C. E., et al. (2012). NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* 488, 531–534. doi: 10.1038/nature11285

Nowak, R. S., and Caldwell, M. M. (1984). A test of compensatory photosynthesis in the field: implications for herbivory tolerance. *Oecologia* 61, 311–318. doi: 10.1007/BF00379627

Nykänen, H., and Koricheva, J. (2004). Damage-induced changes in woody plants and their effects on insect herbivore performance: a meta-analysis. *Oikos* 104, 247–268. doi: 10.1111/j.0030-1299.2004.12768.x

Ohyama, A., and Hirai, M. (1999). Introducing an antisense gene for a cell-wall-bound acid invertase to tomato (*Lycopersicon esculentum*) plants reduces carbohydrate content in leaves and fertility. *Plant Biotech.* 16, 147–151. doi: 10.5511/plantbiotechnology.16.147

Okumoto, S., and Pilot, G. (2011). Amino acid export in plants: a missing link in nitrogen cycling. *Mol. Plant* 4, 453–463. doi: 10.1093/mp/ssr003

Orians, C. M. (2005). Herbivores, vascular pathways and systemic induction: facts and artifacts. *J. Chem. Ecol.* 31, 2231–2242. doi: 10.1007/s10886-005-7099-7

Orians, C. M., Thorn, A., and Gómez, S. (2011). Herbivore-induced resource sequestration in plants: why bother? *Oecologia* 167, 1–9. doi: 10.1007/s00442-011-1968-2

Pageau, K., Reisdorf-Cren, M., Morot-Gaudry, J. F., and Masclaux-Daubresse, C. (2006). The two senescence-related markers, GS1 (cytosolic glutamine synthetase) and GDH (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. *J. Exp. Bot.* 57, 547–557. doi: 10.1093/jxb/erj035

Pate, J. S. (1973). Uptake, assimilation and transport of nitrogen compounds by plants. *Soil Biol. Biochem.* 5, 109–119. doi: 10.1016/0038-0717(73)90097-7

Pate, J. S., Peoples, M. B., and Atkins, C. A. (1983). Post-anthesis economy of carbon in a cultivar of cowpea. *J. Exp. Bot.* 34, 544–562. doi: 10.1093/jxb/34.5.544

Philippe, R. N., Ralph, S. G., Mansfield, S. D., and Bohlmann, J. (2010). Transcriptome profiles of hybrid poplar (*Populus trichocarpa* × *deltoides*) reveal rapid changes in undamaged, systemic sink leaves after simulated feeding by forest tent caterpillar (*Malacosoma disstria*). *New Phytol.* 188, 787–802. doi: 10.1111/j.1469-8137.2010.03392.x

Redak, R. A., and Capinera, J. L. (1994). Changes in western wheatgrass foliage quality following defoliation – consequences for a graminivorous grasshopper. *Oecologia* 100, 80–88. doi: 10.1007/BF00317133

Rehill, B. J. (2001). *The Organismal and Chemical Ecology of a Gall-Forming Aphid on Witch Hazel: A Study of Resource Enhancement by Hormaphis Hamamelidis (Homoptera: Hormaphididae)*. Dissertation, Pennsylvania State University.

Rehill, B. J., and Schultz, J. C. (2012). *Hormaphis hamamelidis* fundatrices benefit by manipulating phenolic metabolism of their host. *J. Chem. Ecol.* 38, 496–498. doi: 10.1007/s10886-012-0115-9

Rennie, E. A., and Turgeon, R. (2009). A comprehensive picture of phloem loading strategies. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14162–14167. doi: 10.1073/pnas.0902279106

Richardson, A. D., Carbone, M. S., Keenan, T. F., Czimczik, C. I., Hollinger, D. Y., Murakami, P., et al. (2013). Seasonal dynamics and age of stemwood nonstructural carbohydrates in temperate forest trees. *New Phytol.* 197, 850–861. doi: 10.1111/nph.12042

Roitsch, T., Balibrea, M. E., Hofmann, M., Proels, R., and Sinha, A. K. (2003). Extracellular invertase: key metabolic enzyme and PR protein. *J. Exp. Bot.* 54, 513–524. doi: 10.1093/jxb/erg050

Roitsch, T., and González, M. C. (2004). Function and regulation of

plant invertases: sweet sensations. *Trends Plant Sci.* 9, 606–613. doi: 10.1016/j.tplants.2004.10.009

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57, 675–709. doi: 10.1146/annurev.arplant.57.032905.105441

Rossato, L., MacDuff, J. H., Laine, P., Le Deunff, E., and Ourry, A. (2002). Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation. *J. Exp. Bot.* 53, 1131–1141. doi: 10.1093/jexbot/53.371.1131

Sagisaka, S. (1993). Amino-acid metabolism in nongrowing environment in higher plants. *Amino Acids* 4, 141–155. doi: 10.1007/BF00805810

Saint-Hilaire, E. G. (1818). *Philosophie Anatomique*, Vol. 2. Paris: J. B. Bailliere, 566.

Sauer, N. (2007). Molecular physiology of higher plant sucrose transporters. *FEBS Lett.* 581, 2309–2317. doi: 10.1016/j.febslet.2007.03.048

Schaarschmidt, S., Roitsch, T., and Hause, B. (2006). Arbuscular mycorrhiza induces gene expression of the apoplastic invertase LIN6 in tomato (*Lycopersicon esculentum*) roots. *J. Exp. Bot.* 57, 4015–4023. doi: 10.1093/jxb/erl172

Schultz, J. C. (1983). Impact of variable plant chemical defenses on insect susceptibility to parasites, predators and diseases. *Symp. Am. Chem. Soc.* 209, 37–55.

Schwachtje, J., and Baldwin, I. T. (2008). Why does herbivore attack reconfigure primary metabolism? *Plant Physiol.* 146, 845–851. doi: 10.1104/pp.107.112490

Schwachtje, J., Minchin, P. E., Jahnke, S., van Dongen, J. T., Schittko, U., and Baldwin, I. T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12935–12940. doi: 10.1073/pnas.0602316103

Shitan, N., Morita, M., and Yazaki, K. (2009). Identification of a nicotine transporter in leaf vacuoles of *Nicotiana tabacum. Plant Signal. Behav.* 4, 530–532. doi: 10.4161/psb.4.6.8588

Shiroya, T., Lister, G. R., Slankis, V., Krotkov, G., and Nelson, C. D. (1966). Seasonal changes in respiration, photosynthesis, and translocation of the ^{14}C labelled products of photosynthesis in young Pinus strobus L. plants. *Ann. Bot.* 30, 81–91.

Simard, S. W. (2009). "Response diversity of mycorrhizas in forest succession following disturbance," in *Mycorrhizas: Functional Processes and Ecological Impacts,* eds C. Azcon-Aguilar, J. M. Barea, S. Gianinazzi, and V. Gianinazzi-Pearson (Heidelberg: Springer-Verlag), Chap. 13, 187–206.

Song, Y. Y., Zeng, R. S., Xu, J. F., Li, J., Shen, X., and Yihdego, W. G. (2010). Interplant communication of tomato plants through underground common mycorrhizal networks. *PLoS ONE* 5:e13324. doi: 10.1371/journal.pone.0013324

Smith, J. P., and Holzapfel, B. P. (2009). Cumulative responses of semillon grapevines to late season perturbation of carbohydrate reserve status. *Am. J. Enol. Vitic.* 60, 461–470.

Staswick, P. E. (1989). Developmental regulation and the influence of plant sinks on vegetative storage protein gene expression in soybean leaves. *Plant Physiol.* 89, 309–315. doi: 10.1104/pp.89.1.309

Steele, L., Caldwell, M., Boettcher, A., and Arnold, T. (2006). Seagrass-pathogen interactions: 'psuedo-induction' of turtlegrass phenolics near wasting disease lesions. *Mar. Ecol. Prog. Ser.* 303, 123–128. doi: 10.3354/meps303123

Steinbrenner, A. D., Gómez, S., Osorio, S., Fernie, A. R., and Orians, C. M. (2011). Herbivore-induced changes in tomato (*Solanum lycopersicum*) primary metabolism: a whole plant perspective. *J. Chem. Ecol.* 37, 1294–1303. doi:10.1007/s10886-011-0042-1

Stepien, V., Sauter, J. J., Martin, F. (1994). Vegetative storage proteins in woody plants. *Plant Physiol. Biochem.* 32, 185–192.

Steppuhn, A., and Baldwin, I. T. (2008). "Induced defenses and the cost-benefit paradigm," in *Induced Plant Resistance to Herbivory*, ed. A. Schaller (Berlin [u.a.]: Springer-Verlag), 61–83. doi: 10.1007/978-1-4020-8182-8_3

Sturm, A., and Chrispeels, M. J. (1990). cDNA cloning of carrot extracellular ßfructosidase and its expression in response to wounding and bacterial infection. *Plant Cell* 2, 1107–1119.

Sturm, A., and Tang, G.-Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends Plant Sci.* 4, 401–407. doi: 10.1016/S1360-1385(99)01470-3

Teskey, R. O., and Hinckley, T. M. (1981). Influence of temperature and water potential on root growth of white oak. *Physiol. Plant.* 52, 363–369. doi: 10.1111/j.1399-3054.1981.tb06055.x

Thorne, J. H., and Rainbird, R. M. (1983). An *in vivo* technique for the study of phloem unloading in seed coats of developing soybean seeds. *Plant Physiol.* 72, 268–71. doi: 10.1104/pp.72.1.268

Thornton, B., and Millard, P. (2006). Effects of severity of defoliation on root functioning in grasses. *J. Range Manage.* 49, 443–447. doi: 10.2307/4002927

Thorpe, M. R., Ferrieri, A. P., Herth, M. M., and Ferrieri, R. A. (2007). 11C-imaging: methyl jasmonate moves in both phloem and xylem, promotes transport of jasmonate, and of photoassimilate even after proton transport is decoupled. *Planta* 226, 541–551. doi: 10.1007/s00425-007-0503-5

Truernit, E., and Sauer, N. (1995). The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H^+ symporter gene directs expression of beta-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. *Planta* 196, 564–570. doi: 10.1007/BF00203657

Turgeon, R. (2010). The puzzle of phloem pressure. *Plant Physiol.* 154, 578–581. doi: 10.1104/pp.110.161679

Turgeon, R., and Gowan, E. (1990). Phloem loading in *Coleus blumei* in the absence of carrier-mediated uptake of export sugar from the apoplast. *Plant Physiol.* 94, 1244–1249. doi: 10.1104/pp.94.3.1244

Turgeon, R., and Wolf, S. (2009). Phloem transport: cellular pathways and molecular trafficking. *Annu. Rev. Plant Biol.* 60, 207–221. doi: 10.1146/annurev.arplant.043008.092045

Vassilyev, A. E. (2010). On the mechanisms of nectar secretion: revisited. *Ann. Bot.* 105, 349–354. doi: 10.1093/aob/mcp302

Walker, T. S., Bais, H. P., Grotewold, E., and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiol.* 132, 44–51. doi: 10.1104/pp.102.019661

Welter, S. C. (1989). "Arthropod impact on plant gas exchange," in *Insect–Plant Interactions,* Vol 1. ed. E. A. Bernays (Boca Raton, FL: CRC Press), 135–150.

Whitham, T. G. (1982). *Individual trees as heterogeneous environments: adaptation to herbivory or epigenetic noise? in Insect Life History Patterns*, eds R. Denno and H. Dingle (Heidelberg: Springer), 9–27.

Williams, C. E., Nemacheck, J. A., Shukle, J. T., Subramanyam, S., Saltzmann, K. D., and Shukle, R. H. (2011). Induced epidermal permeability modulates resistance and susceptibility of wheat seedlings to herbivory by Hessian fly larvae. *J. Exp. Bot.* 62, 4521–4531. doi: 10.1093/jxb/err160

Wink, M., and Roberts, M. F. (1998). "Compartmentation of alkaloid synthesis, transport and storage," in *Alkaloids: Biochemistry, Ecology, and Medicinal Applications*, eds M. F. Roberts and M. Wink (New York: Springer), 239–264.

Wolswinkel, P. (1985). Phloem unloading and turgot-sensitive transport-factors involved in sink control of assimilate partitioning. *Physiol. Plant.* 65, 331–339. doi: 10.1111/j.1399-3054.1985.tb02404.x

Würth, M. K. R., Peláez-Riedl, S., Wright, S. J., and Körner, C. (2005). Non-structural carbohydrate pools in a tropical forest. *Oecologia* 143, 11–24. doi: 10.1007/s00442-004-1773-2

Zhang, L., Cohn, N. S., and Mitchell, J. P. (1996). Induction of a pea cell-wall invertase gene by wounding and its localized expression in phloem. *Plant Physiol.* 112, 1111–1117.

Ziemer, R. R. (1971). Translocation of 14-C in ponderosa pine seedlings. *Can. J. Bot.* 49, 167–171. doi: 10.1139/b71-028

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Regulation of fruit and seed response to heat and drought by sugars as nutrients and signals

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A large body of evidence shows that sugars function both as nutrients and signals to regulate fruit and seed set under normal and stress conditions including heat and drought. Inadequate sucrose import to, and its degradation within, reproductive organs cause fruit and seed abortion under heat and drought. As nutrients, sucrose-derived hexoses provide carbon skeletons and energy for growth and development of fruits and seeds. Sugar metabolism can also alleviate the impact of stress on fruit and seed through facilitating biosynthesis of heat shock proteins (Hsps) and non-enzymic antioxidants (e.g., glutathione, ascorbic acid), which collectively maintain the integrity of membranes and prevent programmed cell death (PCD) through protecting proteins and scavenging reactive oxygen species (ROS). In parallel, sugars (sucrose, glucose, and fructose), also exert signaling roles through cross-talk with hormone and ROS signaling pathways and by mediating cell division and PCD. At the same time, emerging data indicate that sugar-derived signaling systems, including trehalose-6 phosphate (T6P), sucrose non-fermenting related kinase-1 (SnRK), and the target of rapamycin (TOR) kinase complex also play important roles in regulating plant development through modulating nutrient and energy signaling and metabolic processes, especially under abiotic stresses where sugar availability is low. This review aims to evaluate recent progress of research on abiotic stress responses of reproductive organs focusing on roles of sugar metabolism and signaling and addressing the possible biochemical and molecular mechanism by which sugars regulate fruit and seed set under heat and drought.

Keywords: sugar metabolism and signaling, fruit and seed set, heat and drought, cell division, hormones, programmed cell death, reactive oxygen species, non-enzymic antioxidant

INTRODUCTION

Food security is becoming a more and more important and urgent issue with increasing demand for enhancement of crop yield. It is estimated that more than 1 billion people are currently undernourished worldwide (Garrity et al., 2010; Godfray et al., 2010) and the world population is predicted to reach more than 9 billion by 2050 (Fedoroff et al., 2010). To meet this population growth crop yield must be doubled by the middle of this century (Beddington, 2010). Unfortunately, the arable land area for food production is rapidly decreasing because of urbanization, salinization, desertification, and competition from biofuel production (Döös, 2002; Solomon, 2010). This means we have to significantly increase crop yield per unit land area to meet the increasing food demand.

Increasing crop yield is becoming more challenging with global warming. It has been predicted that there will be 1.5–5.8°C increase in average annual temperatures by 2100 (Zinn et al., 2010) and approximately 2.5–16% yield loss for every 1°C increase in temperature above optima (Battisti and Naylor, 2009). Furthermore, in field conditions, drought often occurs simultaneously with heat and the combination of drought and heat can lead to a synergistic detrimental effect on crop productivity (Rampino et al., 2012).

The development of reproductive organs (mainly fruits and seeds and their precursors, ovaries and ovules) plays a dominant role in global crop production. In the top fifteen major crops worldwide, there are 10 crops that are consumed as fruit and seed crops (Ross-Ibarra et al., 2007). Generally, about 75% of the total worldwide crop yield comes from fruit and seed crops (Ruan et al., 2010). Fruit and seed yield is mainly determined by fruit and seed number and their size. Fruit and seed abortion is a major limiting factor for achieving crop yield potential (Boyer and McLaughlin, 2007; Patrick and Stoddard, 2010; Ruan et al., 2012). In the plant life cycle, the early stage of fruit and seed development at the set phase is one of the most sensitive periods to abiotic stresses such as heat, drought, and cold (Barnabás et al., 2008; Hedhly et al., 2008; Thakur et al., 2010). Abiotic stresses during the early reproductive stage often cause abnormal development of reproductive organs that results in failure of fertilization or abortion of fruits and seeds (Thakur et al., 2010), thereby dramatically decreasing crop yield (Setter et al., 2011; Kakumanu et al., 2012). For example, drought at the flowering stage caused severe kernel abortion in maize (McLaughlin and Boyer, 2004a). In tomato, flower abortion rate can reach up to 80% under heat stress (Ruan et al., 2010). Similarly, seed set of *Brassica napus* was reduced by 88% by heat stress (Young et al., 2004). Thus, increasing fruit

and seed set under various abiotic stresses is a viable option for sustaining crop yield in the face of climate change. Despite the importance and sensitivity of the fruit and seed set processes, most research effort in fruit and seed biology to date has been dedicated to the late stage of growth and maturation of fruit and seed (Wang et al., 2009), and little is known about the physiological and molecular mechanisms regulating fruit and seed set under abiotic stresses (Ruan et al., 2012).

SUGAR METABOLISM IN FRUIT AND SEED DEVELOPMENT

Fruit and seed development depends on import of sugars in the form of sucrose transported through phloem from source leaves in most species (Egli, 2010; Foulkes et al., 2011) since fruit photosynthesis is negligible in terms of its contribution of assimilates to fruit development (Blanke and Lenz, 1989). This conclusion is supported by a recent molecular study where the specific suppression of glutamate 1-semialdehyde aminotransferase (GSA) in fruits, a key enzyme in chlorophyll biosynthesis, had no effect on fruit growth and ripening (Lytovchenko et al., 2011). However, fruit photosynthesis plays vital roles in early seed development since seed set was seriously compromised in the transgenic plants, indicating early seed development is more sensitive to reduction of carbon supply than fruit. Before participating in various physiological and metabolic processes, phloem-unloaded sucrose must be degraded into hexoses (glucose and fructose) or their derivates by sucrose synthase (Sus, EC 2.4.1.13) or invertase (INV, EC 3.2.1.26) (Sturm, 1999). Sus is a glycosyl transferase, which reversibly converts sucrose in the presence of UDP into UDP-glucose and fructose, whereas INV irreversibly hydrolyses sucrose into glucose and fructose. Based on their subcellular location, INVs can be classified into three subgroups: cell wall invertase (CWIN), vacuolar invertase (VIN), and cytoplasmic invertase (CIN) (Sturm, 1999).

Sugar metabolism provides not only energy to power numerous cellular processes, but also substrates for biosynthesis of biopolymers such as starch, cellulose, callose, and protein. At the same time, sucrose metabolism in sink organs can help to establish sink strength by lowering sucrose concentration in recipient sink cells thereby facilitating sucrose import from source to sink (Ho, 1988). Furthermore, hexose produced by INV and Sus-mediated sucrose degradation can act as a signaling molecule to regulate plant development (Ruan, 2012). It has been suggested that INV and Sus may play particularly important roles in bulky organs such as fruits and seeds in many crop species as compared to that in their wild progenitors (Xu et al., 2012). For example, the loss of a functional CWIN in maize kernels resulted in a miniature seed phenotype (Miller and Chourey, 1992). Similarly, specific overexpression of CWIN in rice by its native promoter increased grain yield (Wang et al., 2008). In contrast, silencing CWIN expression in tomato resulted in increased fruit abortion, and reduced fruit size and seed number per plant (Zanor et al., 2009).

CENTRAL ROLES OF SUGAR METABOLISM IN FRUIT AND SEED SET UNDER HEAT AND DROUGHT

Compared to vegetative organs, young reproductive organs are less competitive for nutrient acquisition, which may result from their distal location from source leaves, lower transport conductivities through plasmodesmata and differentiating phloem and their low INV activities (Ruan et al., 2012). Thus, even under optimal conditions, sugar availability could become one limiting factor for fruit and seed set (Ghiglione et al., 2008).

Under heat and drought, sugar limitation is a well-known factor leading to fruit and seed abortion (Boyer and McLaughlin, 2007; Barnabás et al., 2008). For example, as a result of heat stress, the growth rate of pollen tubes through the style of cotton is limited by an inadequate supply of sucrose and hexose in the pistil (Snider et al., 2011). In maize, drought resulted in severe ovary abortion, while feeding sucrose to the stems of water-stressed plants partially prevented the abortion and restored kernel number (Zinselmeier et al., 1999; McLaughlin and Boyer, 2004a) demonstrating that low availability of sucrose or hexose is a casual factor of ovary abortion. Further analyses revealed that ovary abortion was linked more closely to the availability of glucose than sucrose in the ovary (McLaughlin and Boyer, 2004a), since sucrose concentrations were completely restored after sucrose feeding but glucose concentrations were only partially restored (Zinselmeier et al., 1999). Therefore, inefficient conversion of sucrose to glucose and fructose is a key limiting step for ovary development under drought. In fact, McLaughlin and Boyer (2004b) showed that sucrose feeding only partially restored the activity of invertase which was decreased under drought. Consistently, silencing of CWIN gene (*Lin5*) in tomato increased fruit abortion under drought (Zanor et al., 2009). On the other hand, elevation in CWIN activity through silencing expression of the CWIN inhibitor in tomato delayed leaf senescence and enhanced fruit and seed development (Jin et al., 2009). It will be important to determine if these CWIN-elevated transgenic plants exhibit increased tolerance to abiotic stress.

POSSIBLE MECHANISMS UNDERLYING REGULATION OF FRUIT AND SEED SET BY SUGARS AS NUTRIENTS UNDER HEAT AND DROUGHT

Sucrose metabolism not only provides energy and carbon skeletons for sink development, but also regulates their response to abiotic stresses by providing hexoses as essential metabolites and signaling molecules (Ruan et al., 2012).

REGULATION OF CARBON PARTITIONING

Mild heat stress before silking enhanced the total biomass of maize plants, but with reduced grain yield (Suwa et al., 2010) which indicates that carbon partitioning favors vegetative organs over reproductive tissues when under stress. The difference in response to abiotic stresses between reproductive and vegetative organs may be related to different responses of sucrose degrading enzymes to stress conditions. For example, drought induced the expression of VIN (*Ivr2*) in vegetative tissues of maize, but reduced the expression of *Ivr2* in reproductive organs (Kim et al., 2000). Similar result was also observed in soybean in which drought decreased the activity of soluble invertase in pods, but not that in leaves (Liu et al., 2004). By using RNA-Seq analysis, Kakumanu et al. (2012) found that there are more genes related to carbohydrate metabolism responsive to drought in the maize

ovary than in young leaves. Among them, one gene encoding sucrose synthase showed decreased expression in the ovary, but not in leaf meristems, under drought. This may decrease the sink strength of these reproductive organs leading to sucrose partitioning in favor of vegetative tissues (Sturm and Tang, 1999; Andersen et al., 2002). Consistent with this postulation, a heat tolerant tomato genotype exhibited higher CWIN and VIN activities in the flower and young fruit and consequently a higher rate of sucrose import into young fruit than a heat sensitive genotype, which collectively contributed to higher fruit set under heat stress (Li et al., 2012). Thus, a high ability of sucrose partitioning to, and its degradation within, reproductive organs could be vital for fruit and seed set under heat and drought.

SUCROSE METABOLISM CONTRIBUTES TO ANTIOXIDANT PROTECTION

Heat and drought often lead to excess accumulation of reactive oxygen species (ROS), including singlet oxygen, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), which may result in oxidative damage of DNA, proteins, and lipids (Oktyabrsky and Smirnova, 2007), and finally programmed cell death (PCD) and fruit and seed abortion (Laloi et al., 2004; Vacca et al., 2004; Foyer and Noctor, 2005). It has been proposed that sugar limitation caused by various stresses might be an important basis for ROS accumulation (Couée et al., 2006; Bolouri-Moghaddam et al., 2010). Glucose metabolism may play positive roles in preventing PCD by scavenging ROS. As the primary carbon and energy source in plants, glucose can feed the oxidative pentose phosphate pathway and produce reducing power for biosynthesis of non-enzymic antioxidants such as glutathione (GSH), ascorbic acid (Asc), phenolic compounds, and flavonoids (Bolouri-Moghaddam et al., 2010), which can efficiently scavenge ROS. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme in the oxidative pentose phosphate pathway. A study on soybean showed that G6PDH plays a central role in maintaining the ROS homeostasis under drought stress through increasing the activities of glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) and the content of GSH and Asc (Liu et al., 2013). In addition, oxidative stresses caused by high light and herbicide, paraquat, can activate antioxidant response in Arabidopsis plants (Sunkar et al., 2006). However, exogenous addition of sucrose to Arabidopsis reduced the expression of SOD genes (*CSD1* and *CSD2*) under high light and paraquat, implying sucrose might prevent oxidant stress to some extent (Dugas and Bartel, 2008). Furthermore, some soluble sugars, such as fructan, might act as ROS scavengers themselves when they exist at high concentration (Van den Ende and Valluru, 2009). Further evidence on sucrose metabolism alleviating oxidative stress comes from transgenic potato plants overexpressing yeast INV that displayed lower malondialdehyde (MDA) content under cold stress (Sinkevich et al., 2010). This indicates INV may improve cold tolerance through enhancing the antioxidant capability of potato plants (Sinkevich et al., 2010). Another example is that oxidative stress such as that caused by application of H_2O_2 often increases the expression of ascorbate peroxidases (APXs), enzymes playing important roles in maintaining the plant's antioxidant system. Overexpressing CIN in Arabidopsis protoplasts, however, alleviates the elevation of APX expression upon H_2O_2 application, which implies that CIN may ameliorate oxidative stress directly (Xiang et al., 2011). Although the majority of these studies were conducted in vegetative tissues, it can be envisaged that sugar metabolism may play positive roles in fruit and seed set through maintaining ROS homeostasis under heat and drought.

SUCROSE METABOLISM AND HEAT SHOCK PROTEINS (Hsps)

Sugar metabolism may also ameliorate effects of abiotic stresses by fueling biosynthesis of heat shock proteins (Hsps). Under heat and drought, Hsps are often induced to prevent or attenuate stress-induced PCD though preserving membrane integrity and protein function. Severe heat stress could lead to the hyperfluidization and disruption of membranes (Horváth et al., 1998; Sangwan et al., 2002), inactivation of proteins by unfolding, misfolding, and aggregation (Sharma et al., 2010) and the accumulation of ROS (Dat et al., 1998; Volkov et al., 2006). These detrimental effects collectively damage photosynthesis and retard plant growth and development and may even cause death of sensitive tissues (Mittler and Blumwald, 2010). During evolution, plants have developed a system of heat-inducible cellular and molecular defences, i.e., the heat stress response (HSR). During HSR, hundreds of specific genes become up-regulated (Mittler et al., 2012). Among them, accumulation of Hsps is a major feature of HSR. Many of the Hsps are chaperones, including Hsp100s, Hsp90s, Hsp70s, Hsp60s, and small Hsps (sHsps) (Finka et al., 2011). Chaperones can prevent protein aggregation or restore misfolded and aggregated proteins to correctly-folded polypeptides. These polypetides can recover their active conformation for normal function after stress exposure (Sharma et al., 2010). Thus, Hsps play an important role in plant heat tolerance. Indeed, constitutive overexpression of Hsp17.7 in carrot (Malik et al., 1999) or Hsp101 in Arabidopsis (Queitsch et al., 2000) increased their heat tolerance.

Studies in animal systems showed that Hsp chaperones (e.g., Hsp70 and sHsps) can block PCD (Beere, 2004; Weiss et al., 2007). In Arabidopsis, *CNGC2* encodes a component of cyclic nucleotide gated Ca^{2+} channels which are a kind of thermosensor in land plants. *CNGC2* mutants accumulated Hsps at lower heat-priming temperatures and consequently have higher heat tolerance and less PCD than wild-type plants (Finka et al., 2012). Surprisingly, *CNGC2* mutants showed growth retardation under control temperature, but without other detectable cellular, morphological, or developmental defects. It has been suggested that this may result from insufficient supply of photoassimilates since maintaining of HSR comes at a high energetic and metabolic cost (Finka et al., 2012; Mittler et al., 2012). Support for this hypothesis comes from a recent finding that heat-stress induced expression of two HSP genes, *LeHSP17.4-CII* and *LeHSP17.6-CII*, correlates with enhanced transcript levels and activities of VIN in 5-d tomato fruit (Li et al., 2012). However, evidence is still lacking as to whether, and to what extent, induction of Hsps is dependent on glucose metabolism and signaling that is coupled with INV activity.

POSSIBLE MECHANISMS UNDERLYING REGULATION OF FRUIT AND SEED SET BY SUGARS AS SIGNALS UNDER HEAT AND DROUGHT

Sucrose metabolism also plays signaling roles in many developmental processes (Ruan, 2012), where sucrose and its degrading products, glucose and fructose, can act as signaling molecules to regulate gene expression (Moore et al., 2003; Wind et al., 2010; Cho and Yoo, 2011). However, it is difficult to distinguish the specific signaling role of sucrose from hexoses since sucrose can be quickly degraded into glucose and fructose. Thus, although the role of sucrose as a signaling molecule in plants was proposed several decades ago, it has been experimentally established and accepted only recently based on emerging evidence (Tognetti et al., 2013). For example, application of exogenous sucrose to the aerial part of Arabidopsis plants facilitate the initiation of lateral roots, but equal molar concentrations of glucose or fructose promote lateral root formation to a much lesser extent (Macgregor et al., 2008). Most recent studies on strawberry (*Fragaria* × *ananassa*) show that sucrose is the key signaling molecule in fruit ripening (Jia et al., 2013). Exogenous sucrose increased ABA content in fruit and accelerated fruit ripening which can be mimicked by turanose, a sucrose non-metabolizable analog, implying the possible signaling role of sucrose in fruit ripening. Their further studies revealed that silencing of the sucrose transporter (*FaSUT1*) by RNAi technique decreased sucrose level and blocked fruit ripening, whereas overexpression of *FaSUT1* increased sucrose level and accelerated fruit ripening (Jia et al., 2013). However, no sucrose-specific sensor has been identified thus far (Wind et al., 2010), and little is known about the nature of sucrose signaling and the regulatory pathways modulated by sucrose.

Recently, it has been suggested that hexoses derived from sucrose cleavage by Sus and INV play important roles in plant development through signaling pathways (Koch, 2004; Ruan, 2012). However, up to now, research on sugar signaling has mainly focused on glucose (Smeekens et al., 2010; Cho and Yoo, 2011). Although fructose is also an abundant hexose produced from both INV- and Sus-catalysed sucrose degradation, little is known about its potential signaling role. Only recently, a systematic study was done in Arabidopsis which revealed that fructose signaling plays an important role in seedling development and fructose-1, 6-biphosphatase was identified as the fructose sensor in the signaling pathway (Cho and Yoo, 2011).

In addition to the above mentioned sugar signaling (i.e., sucrose, glucose, and fructose), the importance of some sugar-derived signaling systems in plant development is becoming increasingly prominent. These systems include the trehalose 6-phosphate (T6P) signal, and the target of rapamycin (TOR) kinase system, the SNF1-related protein kinase (SnRK) and bZIP transcription factor network. Here, we will first focus on the recent progress about the regulatory role of sugar (mainly glucose) signaling in fruit and seed development under abiotic stresses followed by discussion of the possible roles of T6P, TOR, SnRK1, and bZIP in plant development under abiotic stress.

CROSSTALK BETWEEN SUGAR- AND HORMONE-SIGNALING PATHWAYS: A GENERAL PHENOMENON

There is compelling evidence on crosstalk between sugar- and hormone-signaling pathways (LeClere et al., 2010; Ruan, 2012). For example, increased petal and sepal number in tomato in response to inhibition of CWIN gene (*Lin5*) expression is linked to decreased ABA, JA and GA levels (Zanor et al., 2009). Most studies focus on the positive interaction between sugars and auxins, an important hormone in plant development. The Arabidopsis hexokinase (HXK) mutant *gin2* (*glucose insensitive 2*) is less sensitive to exogenous auxin indicating that the glucose-signaling pathway interacts with, or may act downstream of, the auxin signaling pathways through the HXK-dependent pathway (Moore et al., 2003). On the other hand, a recent study on Arabidopsis provides direct evidence that auxin biosynthesis is tightly dependent on endogenous glucose level (Sairanen et al., 2012). Consistently, exogenous glucose up-regulated the expressions of auxin biosynthetic genes (*YUCCA*) and IAA transporter genes (*PINs*) in roots of young Arabidopsis seedlings, and an auxin receptor mutant (*tir1*) and response mutants (*axr2*, *axr3* and *slr1*) showed a defect in glucose-induced root elongation and lateral root production (Mishra et al., 2009). Decrease of hexose level in basal regions of maize kernels by deficiency of CWIN activity results in reduced IAA levels in miniature kernels that are related to suppressed expression of the IAA biosynthesis gene (*ZmYUC*) through a HXK-dependent pathway (LeClere et al., 2010). There is also a positive relationship between sugar and cytokinin signaling. For example, delayed leaf senescence by cytokinin depends on expression of CWIN in tobacco leaves (Lara et al., 2004), which implies a positive relationship between sugar and cytokinin signaling.

A negative interaction exists between glucose and ethylene through ETHYLENE-INSENSITIVE3 (EIN3), a key transcriptional regulator in ethylene signaling (Chao et al., 1997). Glucose can suppress the activity of EINs by enhancing their degradation through ubiquitination. Importantly, the non-signaling glucose analog, 3-*O*-methyl-glucose (3-OMG) which can not be phosphorylated by HXK in plants, did not suppress the activity of EIN3. This finding indicates that glucose acted as a signal molecule to induce the degradation of EIN3 in a HXK dependent manner (Yanagisawa et al., 2003). Indirect evidence also showed a negative interaction between sugars (hexoses) and ABA. For example, increased CWIN activity by silencing expression of CWIN inhibitor (*LeINVINH1*) delayed ABA-induced leaf senescence in tomato, which suggests ABA-mediated senescence is dependent on decreased CWIN activity, hence possibly a lower glucose or fructose level in the apoplasm (Jin et al., 2009). Interestingly, a study on rice anthers showed that ABA accumulation, prior to decreased CWIN and monosaccharide transporter expression, is the initial event responsible for pollen sterility under cold treatment (Oliver et al., 2007). Similarly, an increase in ABA level in maize ovaries occurred immediately after drought was imposed which is followed by a decrease in expression of soluble invertase (*Ivr2*) (Andersen et al., 2002). However, Pinheiro et al. (2011) found that a change in carbohydrate metabolism rather than in ABA level is the initial response of *Lupinus albus* to slowly imposed drought. This contradictory

conclusion may be resulted from variability in type and strength of stress imposed.

Reciprocally, hormones can also affect sugar metabolism. For example, exogenous supplement of NAA to tobacco cells stimulated the activity of CWIN (Weil and Rausch, 1990). The induction of CWIN by cytokinins was observed in suspension cell cultures of *Chenopodium rubrum* (Ehness and Roitsch, 1997), and the result is further supported by the induction of CWIN activity by an endogenous increase of cytokinin in tobacco (Lara et al., 2004). Exogenous ABA decreased the CWIN activity in tomato leaves mainly through increasing the expression of CWIN inhibitor (*INVINH1*) (Jin et al., 2009). Overall, there appears to be synergistic relationships between CWIN activity and growth-related hormones (auxin, cytokinin), but antagonistic relationships between CWIN and senescence hormones (ABA and ethylene).

SUGAR SIGNALING AND CELL DIVISION IN FRUITS AND SEEDS UNDER HEAT AND DROUGHT STRESS

Early evidence about sugar signaling in reproductive organs comes from the observation that hexoses stimulate cell division, while sucrose promotes cell endoreduplication and starch accumulation in *Vicia faba* cotyledons (Weber et al., 1996). These authors further proposed that CWIN in the seed coat of *Vicia faba* can affect the developmental processes of seeds through regulating sugar signaling in the embryo. In maize, mutation of a CWIN gene (*INCW2*) resulted in a miniature seed phenotype by blocking endosperm cell division (Vilhar et al., 2002). Further, Baldet et al. (2006) suggested that the possible mechanism for carbohydrate control of tomato fruit size is through the regulation of cell proliferation. The effect of sugar signaling on cell division in seed and fruit may be realized through their regulation of cyclins (e.g., cyclin D—Dewitte and Murray, 2003; Weber et al., 2005). It has been suggested that decreased glucose level under stress conditions could repress cell division and consequently lead to fruit and seed abortion (Ruan et al., 2012). A recent study showed that drought inhibited the expression of two main invertase genes, *Incw1* and *Incw2*, in the maize ovary and decreased the hexose level (Kakumanu et al., 2012). It has been suggested that this reduced hexose level arrested cell division through decreasing the expression of cyclins and increasing the expression of the cyclin-dependent kinase inhibitor (CDKI). Consistently, in the more resistant leaf meristem, drought increased invertase expression, which maintained the hexose level and cell division activity.

Sugar signaling regulation of cell division might be realized through crosstalk with hormone signaling. It has been proposed that normal fruit and seed development relies on induction of auxin, GA and cytokinin responses and attenuation of ethylene and ABA responses (Dorcey et al., 2009; Ji et al., 2011; Ruan et al., 2012). Auxin, GA and cytokinin can facilitate fruit and seed set and development by enhancing cell division and cell expansion (Gillaspy et al., 1993), whereas ethylene and ABA are senescence and stress hormones which can hamper fruit and seed development (Davies, 2010). It can be postulated that, therefore, under optimal conditions, glucose signals can promote cell division, and consequently fruit and seed set through enhancing growth-related hormone signaling (auxin, GA and cytokinin, **Figure 1A**). Under stress conditions, however, decreased glucose levels resulting from reduced sucrose import into, and INV activity within, ovaries and seeds may lead to activation of senescence hormone signaling (ethylene and ABA) and arrest cell division, and finally fruit and seed abortion (**Figure 1B**).

CROSSTALK BETWEEN SUGAR- AND ROS-SIGNALING PATHWAYS

As detailed above, excess ROS will damage DNA, proteins and lipids. However, ROS also play a signaling role at lower concentrations, which is essential for many developmental or metabolic processes such as PCD, cell wall biosynthesis, and stress responses (Kovtun et al., 2000; Mittler, 2002; Andriunas et al., 2012). An important mechanism of ROS signaling is to regulate the interaction between proteins through altering the redox state of amino acids such as cysteine (Cys). ROS can also play their signaling role through calcium signaling and affecting transcription factors (Munné-Bosch et al., 2013). Among all the ROS species, H_2O_2 is the most common signaling molecule since it is relatively stable and water-soluble (Bienert et al., 2007; Van Breusegem et al., 2008). Under stress conditions, maintaining ROS at an optimal level can facilitate their beneficial signaling function without causing oxidative damage (Liu et al., 2013). As discussed earlier, sugar metabolism plays important roles in maintaining ROS homeostasis. It has also been proposed that there is an interaction between sugar- and ROS-signaling pathways (Bolouri-Moghaddam et al., 2010). Thus, it can be inferred that sugar metabolism may play its signaling role indirectly through interacting with ROS signaling, particularly under stress conditions.

ROLES OF OTHER SUGAR-DERIVED SIGNALING SYSTEMS IN THE ABIOTIC STRESS RESPONSE

TREHALOSE AND TREHALOSE-6-PHOSPHATE

Trehalose is a non-reducing disaccharide sugar, which is synthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). TPS catalyzes the formation of trehalose-6-phosphate (T6P) from glucose-6-phosphate and UDP-glucose, while TPP is responsible for the dephosphorylation of T6P to produce trehalose (Eastmond et al., 2002). Trehalose is ubiquitous in the biosphere, but only in trace amounts in most plants. Although plenty of evidence indicates that trehalose may play important regulatory roles in plant development and resistance to diverse abiotic stresses (Li et al., 2011), emerging evidence has proposed that T6P, rather than trehalose, is the main active component in trehalose metabolism (Eastmond et al., 2002; Schluepmann et al., 2003; O'Hara et al., 2013). Most previous studies on trehalose metabolism only focus on trehalose, but neglect T6P (Almeida et al., 2005; Cortina and Culiáñez-Macià, 2005; Li et al., 2011). As indicated above, T6P is the precursor of trehalose in the biosynthetic pathway, which has lower content than trehalose in plants and thus is hardly detectable. For example, the concentrations of trehalose and T6P in Arabidopsis seedlings are 26.4 and 0.148 nmol g^{-1} FW, respectively (Van Houtte et al., 2013). This may be the most likely reason why many previous studies did not address T6P.

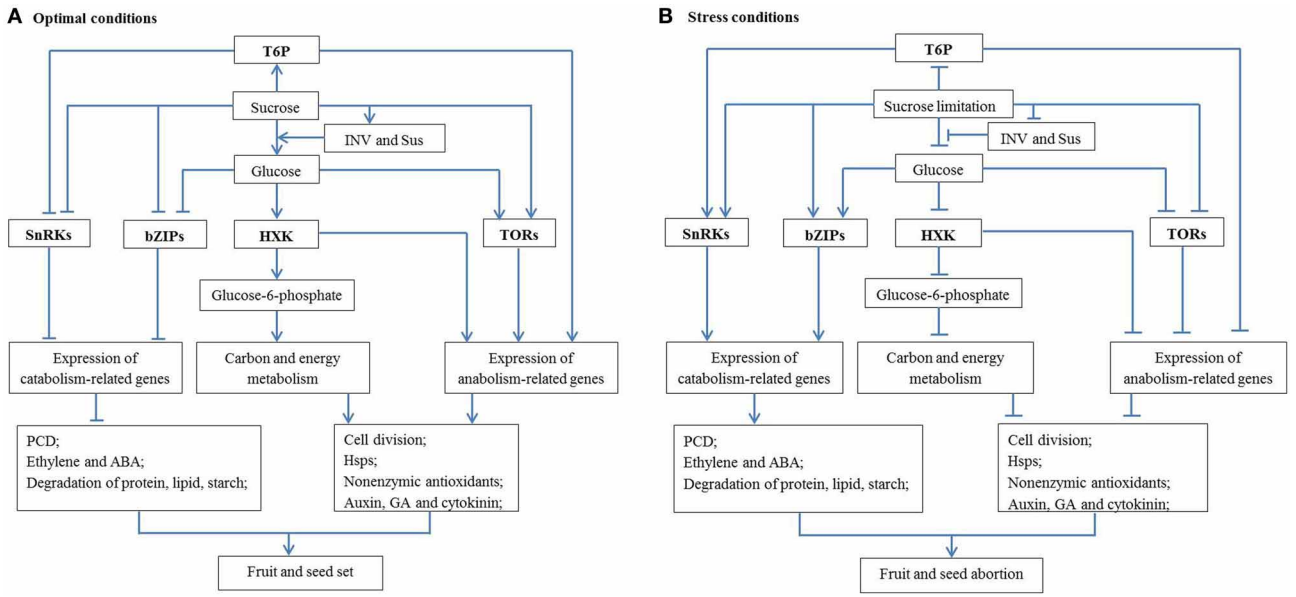

FIGURE 1 | A schematic network illustrating the mechanisms by which sugar metabolism and signaling regulate fruit and seed set under abiotic stresses. (A) Under optimal conditions with high sucrose availability, INV and Sus activities generate sufficient amount of hexoses (glucose) to activate cell division; to facilitate the production of non-enzymatic antioxidants and to inhibit PCD. On the other hand, T6P promotes biosynthetic process and represses the activity of a growth inhibitor, SnRKs. These features interact with hormonal signaling pathways to allow seed and fruit set and subsequent growth to proceed. **(B)** The reverse applies under stress conditions where sugars are limited, and fruits and seeds abort. Arrow and T-type line represent the positive and negative effect, respectively. See text for more details. HSP, heat shock protein; HXK, hexokinase; INV, invertase; PCD, programmed cell death; Sus, sucrose synthase; T6P, trehalose-6-phosphate; TOR, target of rapamycin; SnRK, sucrose non-fermenting-1 (SNF1)-related protein kinases; bZIP, basic leucine-zipper proteins.

Trace amount of T6P in most plants implies that T6P may function as a sugar signal. Most recent studies showed decreased T6P level in Arabidopsis by knocking down the expression of *AtTPS1* led to down-regulated expression of flower-triggering genes (e.g., *FT* and *TSF*) and thus a delay in flowering (Wahl et al., 2013). However, sugar availability (sucrose level) increased in the transgenic plants, indicating T6P acts as a signal to regulate the flower initiation of Arabidopsis (Wahl et al., 2013). Arabidopsis TPS (*AtTPS1*) protein interacted with the cell cycle kinase CDKA;1, suggesting the involvement of trehalose metabolism in cell cycle regulation. T6P may also participate in hormone signaling pathways. For example, increased T6P level down-regulated the expression of *auxin/indole-3-acetic acid* (*Aux/IAA*) and *TIR1* in Arabidopsis, two important genes in auxin sensing and signaling (Paul et al., 2010).

A large body of data showed that trehalose metabolism plays important roles in plant resistance to diverse abiotic stresses (Li et al., 2011). For example, the overexpression of the Arabidopsis TPS gene (*AtTPS1*) gene in tobacco increased tolerance to osmotic stress, drought, desiccation, and temperature stresses (Almeida et al., 2005). The introduction of yeast TPS1 gene into tomato enhanced plant tolerance to drought, salt and oxidative stress (Cortina and Culiáñez-Macià, 2005). Recently, native overexpressing of the rice TPS gene (*OsTPS1*) increased the tolerance of rice seedlings to salinity, drought and cold, possibly through up-regulating the expression of some abiotic stress-related genes including *WSI18*, *RAB16C*, *HSP70*, and *ELIP* (Li et al., 2011). T6P has been proposed to acts as signal molecules to sense carbon availability (O'Hara et al., 2013), a possible mechanism for T6P to regulate plants growth and development in response to abiotic stress. T6P level in plants is positively related to sucrose levels (Schluepmann et al., 2004; Lunn et al., 2006; Martínez-Barajas et al., 2011). Thus, limited sugar availability under various abiotic stresses may reduce the content of T6P. Although no evidence showed there is a direct connection between T6P and the hexokinase-dependent sugar signaling pathway, Schluepmann et al. (2004) suggested there may be a link between T6P and sucrose non-fermenting related kinase-1 (SnRK1), which is known as a inhibitory factor of plant growth (Smeekens et al., 2010). T6P has an inhibitory effect on the activity of SnRK1 (Zhang et al., 2009; Paul et al., 2010). Thus, reduced T6P level under abiotic stresses may lead to an increase in SnRK1 activity and consequently inhibit the biosynthetic processes and plant growth.

TARGET OF RAPAMYCIN

TOR is one member of the family of phosphatidylinositol kinase-related kinases, which is structurally and functionally conserved among organisms and found in nearly all eukaryotes. The TOR signaling has been well studied in fungi and animals (Smeekens et al., 2010) and plays important roles in cell growth and metabolism by integrating the response of cells to growth factors, nutrients, energy, and stress signals (Ahn et al., 2011). Although there is a scarcity of data on TOR in plants, recently it has been

proposed to play an important role in plant development through coordinating development with nutrient availability including sugar (Robaglia et al., 2012). For example, AtTOR protein is indispensable for the normal plant and seed development in Arabidopsis (Deprost et al., 2007). Sugar abundance and starvation activate and inhibit the TOR kinase, respectively, followed by up- and down-regulation of energy-consuming related cellular processes, such as mRNA translation and cell proliferation, respectively (Robaglia et al., 2012). Rapamycin can effectively inhibit Arabidopsis TOR kinase activation by glucose and thus retards glucose-mediated root and leaf growth, indicating the central roles of glucose-TOR signaling in plant development (Xiong and Sheen, 2012). These authors further revealed that glucose-TOR signaling is another glucose signaling pathway in parallel with glucose-HXK and plays important roles in reactivation of the cell cycle in quiescent root meristems of Arabidopsis. This glucose-TOR signaling pathway is decoupled from hormone signaling and it was identified that E2Fa transcription factor is the downstream target of the glucose-TOR signaling pathway, which is responsible for the activation of cell cycle (S-phase) genes after its phosphorylation mediated by TOR kinase (Xiong et al., 2013). Sucrose can also activate this signaling pathway, while fructose can not (Xiong et al., 2013).

SnRKs SIGNALING

Sucrose non-fermenting-1(SNF1)-related protein kinases (SnRKs) is a homolog of the fungal SNF1, which is a global regulator of carbon metabolism in fungi (Coello et al., 2011). Plant SnRKs can be divided into three subgroups: SnRK1s, SnRK2s, and SnRK3s among which SnRK2s and SnRK3s emerged as a result of duplication during plant evolution. It is suggested that SnRK2s and SnRK3s allow plants to link metabolic and stress signaling (Halford and Hey, 2009), which does not occur in mammals and fungi (Coello et al., 2011). This feature gives SnRKs a potential role in regulation of plant responses to stresses. To deal with the sugar limitation (starvation) during stress conditions, plants take measures to limit anabolism and enhance catabolism to save energy and nutrients, in which SnRK act as central regulators (Guérinier et al., 2013). Limited sugar availability activates the activity of SnRKs (Cho et al., 2012), which act as inhibitors of gene expression involved in biosynthetic pathways (Baena-González et al., 2007; Baena-González and Sheen, 2008). At the same time, SnRK1 triggers starch degradation and mobilization under starvation conditions such as darkness (Avila et al., 2012).

In terms of responses to abiotic stresses, loss-of-function of *SOS2*, a SnRK3 gene in Arabidopsis, made plants more sensitive to salt stress, indicating SnRK3 is indispensable for salt tolerance in Arabidopsis (Liu et al., 2000). The knockout or overexpression of SRK2C, an osmotic-stress-activated SnRK2 protein kinase, led to drought-sensitive or tolerant phenotypes, respectively, in Arabidopsis via controlling the expression of stress-related genes (Umezawa et al., 2004). Previous studies showed SnRKs are involved in the ABA signaling pathway, which has been proposed to have a key role in plant tolerance to various abiotic stresses (e.g., salt stress, drought and heat stress) (Coello et al., 2011). For example, the overexpression of *PKABA1*, a SnRK2 gene from barley, mimicked the suppression of ABA to GA-inducible genes in the aleurone layer, indicating a possible role of *PKABA1* in mediating the antagonism between ABA and GA (Gómez-Cadenas et al., 1999). Arabidopsis triple mutant disruptive in three SnRK2s (i.e., SnRK2.2, 2.3, and 2.6) was completely insensitive to ABA treatment (Fujii and Zhu, 2009). SnRK1 has roles in regulation of cell cycle progression in plants. For instance, SnRK1 expression is high in leaf primordia of tomato, but low in meristems (Pien et al., 2001). Most recent studies showed that SnRK1 is involved in the regulation of cell cycle progression in Arabidopsis by controlling the phosphorylation of CDKI p27KIP1 homologs, AtKRP6 and AtKRP7 (Guérinier et al., 2013). The kinase activity of SnRK1 can be inhibited via the phosphorylation by protein kinase AvrPto-dependent Pto-interacting protein3 (Adi3), a suppressor of cell death, indicating SnRK participation in regulation of cell death (Avila et al., 2012).

bZIPs

The basic leucine-zipper (bZIP) proteins are a large family of multifunctional transcription factors which are characterized by a basic DNA binding region and a leucine-zipper coiled-coil motif in eukaryotes (Reinke et al., 2013). Emerging evidence showed that bZIPs play roles in sugar signaling. It has been proposed that Arabidopsis S1 class bZIPs are transducers of sucrose-specific signals (Weltmeier et al., 2009). At the same time, bZIP11 is also a potential target of SnRK1 (O'Hara et al., 2013).

bZIPs can perceive the variation in sugar status of plants and regulate the profile of gene expression, and thus plant response to internal and external signals (Kang et al., 2010). High sugar availability usually down-regulates the activity of bZIP. For example, sucrose inhibited the translation of *AtbZIP11* mRNA in Arabidopsis (Rook et al., 1998). Glucose repressed the transcription of *AtbZIP1* in Arabidopsis via a HK-dependent pathway (Kang et al., 2010). However, glucose repressed the transcription of *AtbZIP63* in Arabidopsis in an HK-independent way (Matiolli et al., 2011). Furthermore, the full repression of *AtbZIP63* expression by glucose at a high concentration (6%) required the participation of ABA, indicating the involvement of *AtbZIP63* in the glucose-ABA interaction network (Matiolli et al., 2011). Thus, it appears that the mechanism of the bZIP inhibition by sugar is complex and varies with changes in sugar species and levels.

Similar to SnRKs, bZIPs also act as growth inhibitory regulators (Smeekens et al., 2010). For example, the overexpression of *AtbZIP1* and *AtbZIP11* halted the seedling growth of Arabidopsis (Hanson et al., 2008; Kang et al., 2010). The growth inhibition from overexpression of bZIP11 can be relieved by T6P accumulation from trehalose feeding (Schluepmann et al., 2004; Delatte et al., 2011). bZIPs are known to be involved in nutrient (e.g., sugar) and/or stress signaling (Kang et al., 2010; Smeekens et al., 2010; O'Hara et al., 2013) to gain a homeostasis between plant growth and nutrient availability, especially under stress conditions. Plenty of evidence has shown that bZIPs play an important role in the response of plants to various stresses. For example, Arabidopsis seedlings overexpressing a pepper bZIP (*CAbZIP1*) showed enhanced resistance to drought and salt stresses, but the phenotype was dwarfed under optimal condition

(Lee et al., 2006), indicating the enhanced stress tolerance may be produced at the cost of normal development. Arabidopsis seedlings expressing soybean bZIP proteins (GmbZIP44, GmbZIP62, or GmbZIP78) exhibited higher tolerance to salt and cold stress, possibly through negative interaction with ABA signaling (Liao et al., 2008). However, rice seedlings overexpressing *OsbZIP52* showed significantly increased sensitivity to cold and drought stress (Liu et al., 2012).

As detailed above, sugar-derived signaling systems (T6P, TOR, SnRK, and bZIP) play important roles in plant responses to abiotic stresses through maintaining the homoeostasis between sugar availability and plant metabolism (Smeekens et al., 2010; Robaglia et al., 2012). Although most of these studies focused on the vegetative stage, the understanding and manipulation of these signaling systems provide new potential options for improvement of seed and fruit set under abiotic stresses including heat and drought.

A POSSIBLE REGULATORY NETWORK UNDERLYING SUGAR-MEDIATED CONTROL OF FRUIT AND SEED SET

The analyses above allow a possible regulatory network to be formulated about how sucrose metabolism and signaling regulate fruit and seed set under abiotic stresses (**Figure 1**). Under optimal conditions, sucrose import and its degradation by INV or Sus produce adequate hexoses for development of fruit and seed (or other sink organs). Glucose signaling can facilitate cell division directly or through promoting the response of growth-related hormones (auxin, GA, and cytokinin) and inhibiting senescence-related hormone pathways, hence allowing fruit and seed set (**Figure 1A**). At the same time, T6P and TORs signaling networks can promote plant growth through up-regulating the expression of anabolism-related genes, whereas SnRKs and bZIP signaling networks with growth inhibitory effects are restrained under optimal conditions. On the other hand, glucose could enter the oxidative pentose phosphate pathway to provide carbon skeletons and energy for biosynthesis of Hsps and non-enzymic antioxidants, thereby preventing oxidative stress and fruit and seed abortion. However, the reverse applies under stress conditions (**Figure 1B**) where a decreased glucose level could lead to hormonal imbalance, excessive ROS and insufficient Hsps, which may arrest cell division and trigger PCD. Furthermore, sugar limitation inhibits activities of T6P and TOR signaling, but induces activities of SnRKs and bZIPs signaling which can also inhibit cell proliferation through suppressing expression of biosynthetic genes. Together, these biochemical and molecular features could lead to seed and fruit abortion or stunted growth under stress conditions (**Figure 1B**).

CONCLUSION AND PERSPECTIVES

A large body of evidence indicates that sugar metabolism and signaling play important regulatory roles in fruit and seed set and their subsequent development. It is clear that more direct molecular evidence is required to dissect the causal and consequential relationship between changes in sugar metabolism and other biochemical processes and associated phenotype. For instance, although it is well known that glucose metabolism affects hormone levels, it remains to be determined whether the impact is exerted through modulating hormonal sensing and signaling or metabolism and how different hormones coordinate to regulate fruit and seed set under stresses. Second, experimental evidence is still lacking as to whether, and to what extent, biosynthesis of Hsps and maintenance of ROS homeostasis are dependent on sugar metabolism and signaling. Third, previous studies mostly focused on the down-regulation of sugar metabolism, which often has multiple detrimental effects on phenotype and consequently masks the key changes in reproductive development (Boyer and McLaughlin, 2007; Zanor et al., 2009). Little is known about what will happen to fruit and seed development under abiotic stress if key sugar metabolic enzymes, INV and Sus, are up-regulated. Fourth, mounting evidence shows that T6P, TOR, SnRK, and bZIP signaling networks play central roles in plant development through integrating sugar availability and developmental programmes to optimize plant performance. However, no sensor has been identified in perception of sugar status in these networks and the molecular functions and the regulatory mechanisms of them are still largely obscure. Thus, more work needs to be dedicated to this field. Finally, the global analysis of stress responsive regulatory pathways by using new techniques such as RNAseq will help us to understand the signaling cascades by which sugar metabolism and signaling regulate fruit and seed response to heat and drought.

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REFERENCES

Almeida, A. M., Villalobos, E., Araújo, S. S., Leyman, B., Van Dijck, P., Alfaro-Cardoso, L., et al. (2005). Transformation of tobacco with an *Arabidopsis thaliana* gene involved in trehalose biosynthesis increases tolerance to several abiotic stresses. *Euphytica* 146, 165–176. doi: 10.1007/s10681-005-7080-0

Andersen, M. N., Asch, F., Wu, Y., Jensen, C. R., Naested, H., Mogensen, V. O., et al. (2002). Soluble invertase expression is an early target of drought stress during the critical, abortion sensitive phase of young ovary development in maize. *Plant Physiol.* 130, 591–604. doi: 10.1104/pp.005637

Andriunas, F. A., Zhang, H. M., Xue, X., Offler, C. E., McCurdy, D. W., and Patrick, J. W. (2012). Reactive oxygen species form part of a regulatory pathway initiating trans-differentiation of epidermal transfer cells in *Vicia faba* cotyledons. *J. Exp. Bot.* 63, 3617–3630. doi: 10.1093/jxb/ers029

Ahn, C. S., Han, J. A., Lee, H. S., Lee, S., and Pai, H. S. (2011). The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants. *Plant Cell* 23, 185–209. doi: 10.1105/tpc.110.074005

Avila, J., Gregory, O. G., Su, D., Deeter, T. A., Chen, S., Silva-Sanchez, C., et al. (2012). The β-subunit of the SnRK1 complex is phosphorylated by the plant cell death suppressor Adi3. *Plant Physiol.* 159, 1277–1290. doi: 10.1104/pp.112.198432

Baena-González, E., Rolland, F., Thevelein, J. M., and Sheen, Jen. (2007). A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448, 938–942.

Baena-González, E., and Sheen, J. (2008). Convergent energy and stress signaling. *Trends Plant Sci.* 13, 474–482. doi: 10.1016/j.tplants.2008.06.006

Baldet, P., Hernould, M., Laporte, F., Mounet, F., Just, D., Mouras, A., et al. (2006). The expression of cell proliferation-related genes in early developing flowers is affected by a fruit load reduction in tomato

plants. *J. Exp. Bot.* 57, 961–970. doi: 10.1093/jxb/erj082

Barnabás, B., Jager, K., and Feher, A. (2008). The effect of drought and heat stress on reproductive processes in cereals. *Plant Cell Environ.* 31, 11–38.

Battisti, D. S., and Naylor, R. L. (2009). Historical warnings of future food insecurity with unprecedented seasonal heat. *Science* 323, 240–244. doi: 10.1126/science.1164363

Beddington, J. (2010). Food security: contributions from science to a new and greener revolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 61–71. doi: 10.1098/rstb.2009.0201

Beere, H. M. (2004). "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *J. Cell Sci.* 117, 2641–2651. doi: 10.1242/jcs.01284

Bienert, G. P., Møller, A. L. B., Kristiansen, K. A., Schulz, A., Møller, I. M., Schjoerring, J. K., et al. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J. Biol. Chem.* 282, 1183–1192. doi: 10.1074/jbc.M603761200

Blanke, M. M., and Lenz, F. (1989). Fruit photosynthesis. *Plant Cell Environ.* 12, 31–46. doi: 10.1111/j.1365-3040.1989.tb01914.x

Bolouri-Moghaddam, M., Le Roy, K., Xiang, L., Rolland, F., and Van den Ende, W. (2010). Sugar signalling and antioxidant network connections in plant cells. *FEBS J.* 277, 2022–2037. doi: 10.1111/j.1742-4658.2010.07633.x

Boyer, J. S., and McLaughlin, J. E. (2007). Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. *J. Exp. Bot.* 58, 267–277. doi: 10.1093/jxb/erl177

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. (1997). Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE 3 and related proteins. *Cell* 89, 1133–1144. doi: 10.1016/S0092-867480300-1

Cho, Y. H., Hong, J. W., Kim, E. C., and Yoo, S. D. (2012). Regulatory functions of SnRK1 in stress-responsive gene expression and in plant growth and development. *Plant Physiol.* 158, 1955–1964. doi: 10.1104/pp.111.189829

Cho, Y. H., and Yoo, S. D. (2011). Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *PLoS Genet.* 7:1001263. doi: 10.1371/journal.pgen.1001263

Coello, P., Hey, S. J., and Halford, N. G. (2011). The sucrose non-fermenting-1-related (SnRK) family of protein kinases: potential for manipulation to improve stress tolerance and increase yield. *J. Exp. Bot.* 62, 883–893. doi: 10.1093/jxb/erq331

Cortina, C., and Culiáñez-Macià, F. A. (2005). Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. *Plant Sci.* 169, 75–82.

Couée, I., Sulmon, C., Gouesbet, G., and El Amrani, A. (2006). An involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J. Exp. Bot.* 57, 449–459. doi: 10.1093/jxb/erj027

Dat, J. F., Lopez-Delgado, H., Foyer, C. H., and Scott, I. M. (1998). Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. *Plant Physiol.* 116, 1351–1357. doi: 10.1104/pp.116.4.1351

Davies, P. J. (2010). "The plant hormones: their nature, occurrence, and functions" in *Plant Hormones: Biosynthesis, Signal Transduction, Action!* ed P. Davies (Dordrecht: Springer Press), 1–15.

Delatte, T. L., Sedijani, P., Kondou, Y., Matsui, M., de Jong, G. J., Somsen, G. W., et al. (2011). Growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway. *Plant Physiol.* 157, 160–174. doi: 10.1104/pp.111.180422

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Nicolai, M., et al. (2007). The *Arabidopsis* TOR kinase links plant growth, yield, stress resistance and mRNA translation. *EMBO Rep.* 8, 864–870. doi: 10.1038/sj.embor.7401043

Dewitte, W., and Murray, J. A. H. (2003). The plant cell cycle. *Annu. Rev. Plant Biol.* 54, 235–264. doi: 10.1146/annurev.arplant.54.031902.134836

Dorcey, E., Urbez, C., Blázquez, M. A., Carbonell, J., and Perez-Amador, M. A. (2009). Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in *Arabidopsis*. *Plant J.* 58, 318–332. doi: 10.1111/j.1365-313X.2008.03781.x

Döös, B. R. (2002). Population growth and loss of arable land. *Glob. Environ. Change* 12, 303–311. doi: 10.1016/S0959-378000043-2

Dugas, D. V., and Bartel, B. (2008). Sucrose induction of *Arabidopsis* miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol. Biol.* 67, 403–417. doi: 10.1007/s11103-008-9329-1

Eastmond, P. J., Van Dijken, A. J. H., Spielman, M., Kerr, A., Tissier, A., Dickinson, H. G., et al. (2002). Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. *Plant J.* 29, 225–235. doi: 10.1046/j.1365-313x.2002.01220.x

Egli, D. B. (2010). SOYPOD: a model of fruit set in soybean. *Agron. J.* 102, 39–47. doi: 10.2134/agronj2009.0222

Ehness, R., and Roitsch, T. (1997). Coordinated induction of mRNAs for extracellular invertase and a glucose transporter in *Chenopodium rubrum* by cytokinins. *Plant J.* 11, 539–548. doi: 10.1046/j.1365-313X.1997.11030539.x

Fedoroff, N. V., Battisti, D. S., Beachy, R. N., Cooper, P. J. M., Fischhoff, D. A., Hodges, C. N., et al. (2010). Radically rethinking agriculture for the 21st century. *Science* 327, 833–834. doi: 10.1126/science.1186834

Finka, A., Mattoo, R. U. H., and Goloubinoff, P. (2011). Meta-analysis of heat- and chemically upregulated chaperone genes in plant and human cells. *Cell Stress Chaperones* 16, 15–31. doi: 10.1007/s12192-010-0216-8

Finka, A., Quendet, A. F. H., Maathuis, F. J. M., Saidi, Y., and Goloubinoff, P. (2012). Plasma membrane cyclic nucleotide gated calcium channels control land plant thermal sensing and acquired thermotolerance. *Plant Cell* 24, 3333–3348. doi: 10.1105/tpc.112.095844

Foulkes, M. J., Slafer, G. A., Davies, W. J., Berry, P. M., Sylvester-Bradley, R., Martre, P., et al. (2011). Raising yield potential of wheat. III. optimizing partitioning to grain while maintaining lodging resistance. *J. Exp. Bot.* 62, 469–486. doi: 10.1093/jxb/erq300

Foyer, C. H., and Noctor, G. (2005). Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ.* 28, 1056–1071. doi: 10.1111/j.1365-3040.2005.01327.x

Fujii, H., and Zhu, J. K. (2009). *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl. Acad. Sci. U.S.A.* 1106, 8380–8385. doi: 10.1073/pnas.0903144106

Garrity, D. P., Akinnifesi, F. K., Ajayi, O. C., Weldesemayat, S. G., Mowo, J. G., Kalinganire, A., et al. (2010). Evergreen agriculture: a robust approach to sustainable food security in africa. *Food Secur.* 2, 197–214. doi: 10.1007/s12571-010-0070-7

Ghiglione, H. O., Gonzalez, F. G., Serrago, R., Maldonado, S. B., Chilcott, C., Curá, J. A., et al. (2008). Autophagy regulated by day length determines the number of fertile florets in wheat. *Plant J.* 55, 1010–1024. doi: 10.1111/j.1365-313X.2008.03570.x

Gillaspy, G., Ben-David, H., and Gruissem, W. (1993). Fruits: a developmental perspective. *Plant Cell* 5, 1439–1451.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., et al. (2010). Food security: the challenge of feeding 9 billion people. *Science* 327, 812–818. doi: 10.1126/science.1185383

Gómez-Cadenas, A., Verhey, S. D., Holappa, L. D., Shen, Q., Ho, T.-H. D., and Walker-Simmons, M. K. (1999). An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid-suppressed gene expression in barley aleurone layers. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1767–1772. doi: 10.1073/pnas.96.4.1767

Guérinier, T., Millan, L., Crozet, P., Oury, C., Rey, F., Valot, B., et al. (2013). Phosphorylation of p27KIP1 homologs KRP6 and 7 by SNF1-related protein kinase-1 links plant energy homeostasis and cell proliferation. *Plant J.* doi: 10.1111/tpj.12218

Halford, N. G., and Hey, S. J. (2009). SNF1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. *Biochem. J.* 419, 247–259. doi: 10.1042/BJ20082408

Hanson, J., Hanssen, M., Wiese, A., Hendriks, M. M., and Smeekens, S. (2008). The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of ASPARAGINE SYNTHETASE1 and PROLINE DEHYDROGENASE2. *Plant J.* 53, 935–949. doi: 10.1111/j.1365-313X.2007.03385.x

Hedhly, A., Hormaza, J. I., and Herrero, M. (2008). Global warming and plant sexual reproduction. *Trends Plant Sci.* 14, 30–36. doi: 10.1016/j.tplants.2008.11.001

Ho, L. C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39, 355–378.

doi: 10.1146/annurev.pp.39.060188.002035

Horváth, I., Glatz, A., Varvasovszki, V., Török, Z., Páli, T., Balogh, G., et al. (1998). Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: Identification of *hsp17* as a "fluidity gene". *Proc. Natl. Acad. Sci. U.S.A.* 95, 3513–3518. doi: 10.1073/pnas.95.7.3513

Ji, X., Dong, B., Shiran, B., Talbot, M. J., Edlington, J. E., Hughes, T., et al. (2011). Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant Physiol.* 156, 647–662. doi: 10.1104/pp.111.176164

Jia, H., Wang, Y., Sun, M., Li, B., Han, Y., Zhao, Y., et al. (2013). Sucrose functions as a signal involved in the regulation of strawberry fruit development and ripening. *New Phytol.* 198, 453–465. doi: 10.1111/nph.12176

Jin, Y., Ni, D. A., and Ruan, Y. L. (2009). Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level. *Plant Cell* 21, 2072–2089. doi: 10.1105/tpc.108.063719

Kakumanu, A., Ambavaram, M. M. R., Klumas, C., Krishnan, A., Batlang, U., Myers, E., et al. (2012). Effects of drought on gene expression in maize reproductive and leaf meristem tissue revealed by RNA-Seq. *Plant Physiol.* 160, 846–867. doi: 10.1104/pp.112.200444

Kang, S. G., Price, J., Lin, P. C., Hong, J. C., and Jang, J. C. (2010). The *Arabidopsis* bZIP1 transcription factor is involved in sugar signaling, protein networking, and DNA binding. *Mol. Plant* 3, 361–373. doi: 10.1093/mp/ssp115

Kim, J. Y., Mahé, A., Brangeon, J., and Prioul, J. L. (2000). A maize vacuolar invertase, *IVR*$_2$, is induced by water stress: organ/tissue specificity and diurnal modulation of expression. *Plant Physiol.* 124, 71–84. doi: 10.1104/pp.124.1.71

Koch, K. E. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Curr. Opin. Plant Biol.* 7, 235–246. doi: 10.1016/j.pbi.2004.03.014

Kovtun, Y., Chiu, W. L., Tena, G., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2940–2945. doi: 10.1073/pnas.97.6.2940

Laloi, C., Apel, K., and Danon, A. (2004). Reactive oxygen signaling: the latest news. *Curr. Opin. Plant Biol.* 7, 323–328. doi: 10.1016/j.pbi.2004.03.005

Lara, M. E. B., Garcia, M. C. G., Fatima, T., Ehneb, R., Lee, T. K., Proels, R., et al. (2004). Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. *Plant Cell* 16, 1276–1287. doi: 10.1105/tpc.018929

LeClere, S., Schmelz, E. A., and Chourey, P. S. (2010). Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. *Plant Physiol.* 153, 306–318. doi: 10.1104/pp.110.155226

Lee, S. C., Choi, H. W., Hwang, I. S., Choi, D. S., and Hwang, B. K. (2006). Functional roles of the pepper pathogen-induced bZIP transcription factor, CAbZIP1, in enhanced resistance to pathogen infection and environmental stresses. *Planta* 224, 1209–1225. doi: 10.1007/s00425-006-0302-4

Li, H. W., Zang, B. S., Deng, X. W., and Wang, X. P. (2011). Overexpression of the trehalose-6-phosphate synthase gene *OsTPS1* enhances abiotic stress tolerance in rice. *Planta* 234, 1007–1018. doi: 10.1007/s00425-011-1458-0

Li, Z., Palmer, W. M., Martin, A. P., Wang, R., Rainsford, F., Jin, Y., et al. (2012). High invertase activity in tomato reproductive organs correlates with enhanced sucrose import into, and heat tolerance of, young fruit. *J. Exp. Bot.* 63, 1155–1166. doi: 10.1093/jxb/err329

Liao, Y., Zou, H. F., Wei, W., Hao, Y. J., Tian, A. G., Huang, J., et al. (2008). Soybean GmbZIP44, GmbZIP62 and GmbZIP78 genes function as negative regulator of ABA signaling and confer salt and freezing tolerance in transgenic *Arabidopsis*. *Planta* 228, 225–240.

Liu, J., Ishitani, M., Halfter, U., Kim, C. S., and Shu, J. K. (2000). The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3730–3734

Liu, C., Wu, Y., and Wang, X. (2012). bZIP transcription factor OsbZIP52/RISBZ5: a potential negative regulator of cold and drought stress response in rice. *Planta* 235, 1157–1169.

Liu, J., Wang, X., Hu, Y., Hu, W., and Bi, Y. (2013). Glucose-6-phosphate dehydrogenase plays a pivotal role in tolerance to drought stress in soybean roots. *Plant Cell Rep.* 32, 415–429. doi: 10.1007/s00299-012-1374-1

Liu, F., Jensen, C. R., and Andersen, M. N. (2004). Drought stress effect on carbohydrate concentration in soybean leaves and pods during early reproductive development, its implication in altering pod set. *Field Crops Res.* 86, 1–13. doi: 10.1016/S0378-429000165-5

Lunn, J. E., Feil, R., Hendriks, J. H. M., Gibon, Y., Morcuende, R., Osuna, D., et al. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochem. J.* 397(Pt 1), 139–148

Lytovchenko, A., EickmeierPons, C., Osorio, S., Szecowka, M., Lehmberg, K., Arrivault, S., et al. (2011). Tomato fruit photosynthesis is seemingly unimportant in primary metabolism and ripening but plays a considerable role in seed development. *Plant Physiol.* 157, 1650–1663. doi: 10.1104/pp.111.186874

Macgregor, D. R., Deak, K. I., Ingram, P. A., and Malamy, J. E. (2008). Root system architecture in *Arabidopsis* grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell* 20, 2643–2660. doi: 10.1105/tpc.107.055475

Malik, M. K., Slovin, J. P., Hwang, C. H., and Zimmerman, J. L. (1999). Modified expression of a carrot small heat shock protein gene, *hsp17.7*, results in increased or decreased thermotolerance double dagger. *Plant J.* 20, 89–99. doi: 10.1046/j.1365-313X.1999.00581.x

Martínez-Barajas, E., Delatte, T., Schluepmann, H., de Jong, G. J., Somsen, G. W., Nunes, C., et al. (2011). Wheat grain development is characterized by remarkable trehalose 6-phosphate accumulation pregrain filling: tissue distribution and relationship to SNF1-related protein kinase1 activity. *Plant Physiol.* 156, 373–381. doi: 10.1104/pp.111.174524

Matiolli, C. C., Tomaz, J. P., Duarte, G. T., Prado, F. M., Del Bem, L. E. V., Silveira, A. B., et al. (2011). The *Arabidopsis* bZIP gene AtbZIP63 is a sensitive integrator of transient abscisic acid and glucose signals. *Plant Physiol.* 157, 692–705. doi: 10.1104/pp.111.181743

McLaughlin, J. E., and Boyer, J. S. (2004a). Glucose localisation in maize ovaries when kernel number decreases at low water potential and sucrose is fed to the stems. *Ann. Bot.* 94, 75–86.

McLaughlin, J. E., and Boyer, J. S. (2004b). Sugar-responsive gene expression, invertase activity, and senescence in aborting maize ovaries at low water potentials. *Ann. Bot.* 94, 675–689.

Miller, M. E., and Chourey, P. S. (1992). The maize invertase-deficient *minature-1* seed mutation is associated with aberrant pedicel and endosperm development. *Plant Cell* 4, 297–305.

Mishra, B. S., Singh, M., Aggrawal, P., and Laxmi, A. (2009). Glucose and auxin signalling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. *PLoS ONE* 4:e4502. doi: 10.1371/journal.pone.0004502

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405–410. doi: 10.1016/S1360-138502312-9

Mittler, R., and Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. *Annu. Rev. Plant Biol.* 61, 443–462. doi: 10.1146/annurev-arplant-042809-112116

Mittler, R., Finka, A., and Goloubinoff, P. (2012). How do plants feel the heat. *Trends Biochem. Sci.* 37, 118–125

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W. H., Liu, Y. X., et al. (2003). Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* 300, 332–336. doi: 10.1126/science.1080585

Munné-Bosch, S., Queval, G., and Foyer, C. H. (2013). The impact of global change factors on redox signaling underpinning stress tolerance. *Plant Physiol.* 161, 5–19. doi: 10.1104/pp.112.205690

O'Hara, L. E., Paul, M. J., and Wingler, A. (2013). How do sugars regulate plant growth and development. New insight into the role of trehalose-6-phosphate. *Mol. Plant.* 6, 261–274. doi: 10.1093/mp/sss120

Oktyabrsky, O. N., and Smirnova, G. V. (2007). Redox regulation of cellular functions. *Biochemistry (Mosc.)* 72, 132–145. doi: 10.1134/S0006297907020022

Oliver, S. N., Dennis, E. S., and Dolferus, R. (2007). ABA regulates apoplastic sugar transport and is a potential signal for cold-induced pollen sterility in rice. *Plant Cell Physiol.* 48, 1319–1330. doi: 10.1093/pcp/pcm100

Patrick, J. W., and Stoddard, F. L. (2010). Physiology of flowering and grain filling in faba bean.

Field Crops Res. 115, 234–242. doi: 10.1016/j.fcr.2009.06.005

Paul, M. J., Jhurreea, D., Zhang, Y., Primavesi, L. F., Delatte, T., Schluepmann, H., et al. (2010). Up-regulation of biosynthetic processes associated with growth by trehalose 6-phosphate. *Plant Signal. Behav.* 5, 1–7. doi: 10.4161/psb.5.4.10792

Pien, S., Wyrzykowska, J., and Fleming, A. J. (2001). Novel marker genes for early leaf development indicate spatial regulation of carbohydrate metabolism within the apical meristem. *Plant J.* 25, 663–674. doi: 10.1046/j.1365-313x.2001.01002.x

Pinheiro, C., António, C., Ortuño, M. F., Dobrev, P. I., Hartung, W., and Thomas-Oates, J. (2011). Initial water deficit effects on *Lupinus albus* photosynthetic performance, carbon metabolism, and hormonal balance: metabolic reorganization prior to early stress responses. *J. Exp. Bot.* 62, 4965–4974.

Queitsch, C., Hong, S. W., Vierling, E., and Lindquist, S. (2000). Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. *Plant Cell* 12, 479–492.

Rampino, P., Mita, G., Fasano, P., Borrelli, G. M., Aprile, A., Dalessandro, G., et al. (2012). Novel durum wheat genes up-regulated in response to a combination of heat and drought stress. *Plant Physiol. Biochem.* 56, 72–78. doi: 10.1016/j.plaphy.2012.04.006

Reinke, A. W., Baek, J., Ashenberg, O., and Keating, A. E. (2013). Networks of bZIP protein-protein interactions diversified over a billion years of evolution. *Science* 340, 730–734.

Robaglia, C., Thomas, M., and Meyer, C. (2012). Sensing nutrient and energy status by SnRK1 and TOR kinases. *Curr. Opin. Plant Biol.* 15, 301–307. doi: 10.1016/j.pbi.2012.01.012

Rook, F., Gerrits, N., Kortstee, A., vanKampen, M., Borrias, M., Weisbeek, P., et al. (1998). Sucrose-specific signalling represses translation of the *Arabidopsis* ATB2 bZIP transcription factor gene. *Plant J.* 15, 253–263. doi: 10.1046/j.1365-313X.1998.00205.x

Ross-Ibarra, J., Morrell, P. L., and Gaut, B. S. (2007). Plant domestication, a unique opportunity to identify the genetic basis of adaptation. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8641–8648. doi: 10.1073/pnas.0700643104

Ruan, Y. L., Jin, Y., Li, G. J., Yang, Y. J., and Boyer, J. S. (2010). Sugar input, metabolism and signaling mediated by invertase: roles in development, yield potential and response to drought and heat. *Mol. Plant* 3, 942–955. doi: 10.1093/mp/ssq044

Ruan, Y. L. (2012). Signaling role of sucrose metabolism in development. *Mol. Plant* 5, 763–765. doi: 10.1093/mp/sss046

Ruan, Y. L., Patrick, J. W., Bouzayen, M., Osorio, S., and Fernie, A. R. (2012). Molecular regulation of seed and fruit set. *Trends Plant Sci.* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Sairanen, I., Novák, O., Pìnèík, A., Ikeda, Y., Jones, B., Sandberg, G., et al. (2012). Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in *Arabidopsis*. *Plant Cell* 24, 4907–4916. doi: 10.1105/tpc.112.104794

Sangwan, V., Orvar, B. L., Beyerly, J., Hirt, H., and Dhindsa, R. S. (2002). Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *Plant J.* 31, 629–638. doi: 10.1046/j.1365-313X.2002.01384.x

Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S., and Paul, M. (2003). Trehalose 6-phosphate is indispensible for carbohydrate utilization and growth in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 6849–6854

Schluepmann, H., van Dijken, A., Aghdasi, M., Wobbes, B., Paul, M., and Smeekens, S. (2004). Trehalose mediated growth inhibition of *Arabidopsis* seedlings is due to trehalose-6-phosphate accumulation. *Plant Physiol.* 135, 879–890. doi: 10.1104/pp.104.039503

Setter, T. L., Yan, J., Warburton, M., Ribaut, J. M., Xu, Y., Sawkins, M., et al. (2011). Genetic association mapping identifies single nucleotide polymorphisms in genes that affect abscisic acid levels in maize floral tissues during drought. *J. Exp. Bot.* 62, 701–716. doi: 10.1093/jxb/erq308

Sharma, S. K., De los Rios, P., Christen, P., Lustig, A., and Goloubinoff, P. (2010). The kinetic parameters and energy cost of the Hsp70 chaperone as a polypeptide unfoldase. *Nat. Chem. Biol.* 6, 914–920. doi: 10.1038/nchembio.455

Sinkevich, M. S., Naraykina, N. V., and Trunova, T. I. (2010). Involvement of sugars in the antioxidant defense against paraquat-induced oxidative stress in potato transformed with yeast invertase gene. *Doklady Biol. Sci.* 434, 338–340. doi: 10.1134/S0012496610050133

Smeekens, S., Ma, J., Hanson, J., and Rolland, F. (2010). Sugar signals and molecular networks controlling plant growth. *Curr. Opin. Plant Biol.* 13, 274–279. doi: 10.1016/j.pbi.2009.12.002

Snider, J. L., Oosterhuis, D. M., Loka, D. A., and Kawakami, E. M. (2011). High temperature limits *in vivo* pollen tube growth rates by altering diurnal carbohydrate balance in field-grown *Gossypium hirsutum* pistils. *J. Plant Physiol.* 168, 1168–1175. doi: 10.1016/j.jplph.2010.12.011

Solomon, B. D. (2010). Biofuels and sustainability. *Ann. N.Y. Acad. Sci.* 1185, 119–134. doi: 10.1111/j.1749-6632.2009.05279.x

Sturm, A. (1999). Invertases: primary structures, functions and roles in plant development and sucrose partitioning. *Plant Physiol.* 121, 1–7. doi: 10.1104/pp.121.1.1

Sturm, A., and Tang, G. Q. (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends Plant Sci.* 4, 401–407. doi: 10.1016/S1360-138501470-3

Sunkar, R., Kapoor, A., and Zhu, J. K. (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18, 2051–2065. doi: 10.1105/tpc.106.041673

Suwa, R., Hakata, H., Hara, H., El-Shemy, H. A., Adu-Gyamfi, J. J., Nguyen, N. T., et al. (2010). High temperature effects on photosynthate partitioning and sugar metabolism during ear expansion in maize (*Zea mays* L.) genotypes. *Plant Physiol. Biochem.* 48, 124–130. doi: 10.1016/j.plaphy.2009.12.010

Thakur, P., Kumar, S., Malik, J. A., Berger, J. D., and Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: an overview. *Environ. Exp. Bot.* 67, 429–443. doi: 10.1016/j.envexpbot.2009.09.004

Tognetti, J. A., Pontis, H. G., and Martínez-Noël, G. M. A. (2013). Sucrose signaling in plants: a world yet to be explored. *Plant Signal. Behav.* 8:e23316. doi: 10.4161/psb.23316

Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004). SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-response gene expression in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A* 101, 17306–17311. doi: 10.1073/pnas.0407758101

Vacca, R. A., de Pinto, M. C., Valenti, D., Passarella, S., Marra, E., and De Gara, L. (2004). Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. *Plant Physiol.* 134, 1100–1112. doi: 10.1104/pp.103.035956

Van Breusegem, F., Bailey-Serres, J., and Mittler, R. (2008). Unraveling the tapestry of networks involving reactive oxygen species in plants. *Plant Physiol.* 147, 978–984. doi: 10.1104/pp.108.122325

Van den Ende, W., and Valluru, R. (2009). Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging. *J. Exp. Bot.* 60, 9–18. doi: 10.1093/jxb/ern297

Van Houtte, H., Vandesteene, L., López-Galvis, L., Lemmens, L., Kissel, E., Carpentier, S., et al. (2013). Overexpression of the trehalase gene AtTRE1 leads to increased drought stress tolerance in *Arabidopsis* and is involved in abscisic acid-induced stomatal closure. *Plant Physiol.* 161, 1158–1171. doi: 10.1104/pp.112.211391

Vilhar, B., Kladnik, A., Blejec, A., Chourey, P. S., and Dermastia, M. (2002). Cytometrical evidence that the loss of seed weight in the miniature1 seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. *Plant Physiol.* 129, 23–30. doi: 10.1104/pp.001826

Volkov, R. A., Panchuk, I. I., Mullineaux, P. M., and Schöffl, F. (2006). Heat stress-induced H_2O_2 is required for effective expression of heat shock genes in *Arabidopsis*. *Plant Mol. Biol.* 61, 733–746. doi: 10.1007/s11103-006-0045-4

Wahl, V., Ponnu, J., Schlereth, A., Stéphanie, A., Langenecker, T., Franke, A., et al. (2013). Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science* 339, 704–707. doi: 10.1126/science.1230406

Wang, E., Wang, J., Zhu, X., Hao, W., Wang, L., Li, Q., et al. (2008). Control of rice grain-filling and yield by a gene with a potential signature of domestication. *Nat. Genet.* 40, 1370–1374. doi: 10.1038/ng.220

Wang, H., Schauer, N., Usadel, B., Frasse, P., Zouine, M., Hernould, M., et al. (2009). Regulatory features underlying pollination-dependent and -independent tomato fruit set revealed by transcript and primary metabolite profiling. *Plant Cell* 21, 1428–1452. doi: 10.1105/tpc.108.060830

Weber, H., Borisjuk, L., and Wobus, U. (1996). Controlling seed

development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. *Plant J.* 10, 823–834.

Weber, H., Borisjuk, L., and Wobus, U. (2005). Molecular physiology of legume seed development. *Annu. Rev. Plant Biol.* 56, 253–279. doi: 10.1146/annurev.arplant.56.032604.144201

Weil, M., and Rausch, T. (1990). Cell wall invertase in tobacco crown gall cells: enzyme properties and regulation by auxin. *Plant Physiol.* 94, 1575–1581. doi: 10.1104/pp.94.4.1575

Weiss, Y. G., Bromberg, Z., Raj, N., Raphael, J., Goloubinoff, P., Ben-Neriah, Y., et al. (2007). Enhanced heat shock protein 70 expression alters proteasomal degradation of IkappaB kinase in experimental acute respiratory distress syndrome. *Crit. Care Med.* 35, 2128–2138. doi: 10.1097/01.CCM.0000278915.78030.74

Weltmeier, F., Rahmani, F., Ehlert, A., Dietrich, K., Schutze, K., Wang, X., et al. (2009). Expression patterns within the *Arabidopsis* C/S1 bZIP transcription factor network: availability of heterodimerization partners controls gene expression during stress response and development. *Plant Mol. Biol.* 69, 107–119. doi: 10.1007/s11103-008-9410-9

Wind, J., Smeekens, S., and Hanson, J. (2010). Sucrose: metabolite and signaling molecule. *Phytochemistry* 71, 1610–1614. doi: 10.1016/j.phytochem.2010.07.007

Xiang, L., Li, Y., Rolland, F., and Van den Ende, W. (2011). Neutral invertase, hexokinase and mitochondrial ROS homeostasis: emerging links between sugar metabolism, sugar signaling and ascorbate synthesis. *Plant Signal. Behav.* 6, 1567–1573. doi: 10.4161/psb.6.10.17036

Xiong, Y., McCormack, M., Li, L., and Sheen, J. (2013). Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* 496, 181–186.

Xiong, Y., and Sheen, J. (2012). Rapamycin and glucose-target of rapamycin (TOR) protein signaling in plants. *J. Biol. Chem.* 287, 2836–2842. doi: 10.1074/jbc.M111.300749

Xu, S., Brill, E., Llewellyn, D., Furbank, R. T., and Ruan, Y. L. (2012). Over-expression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion and enhances fiber production. *Mol. Plant* 5, 430–441. doi: 10.1093/mp/ssr090

Yanagisawa, S., Yoo, S. D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. *Nature* 425, 521–525. doi: 10.1038/nature01984

Young, L. W., Wilen, R. W., and Bonham-Smith, P. C. (2004). High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production. *J. Exp. Bot.* 55, 485–495. doi: 10.1093/jxb/erh038

Zanor, M. I., Osorio, S., Nunes-Nesi, A., Carrari, F., Lohse, M., Usadel, B., et al. (2009). RNA interference of LIN5 in tomato confirms its role in controlling brix content, uncovers the influence of sugars on the levels of fruit hormones and demonstrates the importance of sucrose cleavage for normal fruit development and fertility. *Plant Physiol.* 150, 1204–1218. doi: 10.1104/pp.109.136598

Zhang, Y., Primavesi, L. F., Jhurreea, D., Andralojc, P. J., Mitchell, R. A. C., Powers, S. J., et al. (2009). Inhibition of Snf1- related protein kinase (SnRK1) activity and regulation of metabolic pathways by trehalose 6-phosphate. *Plant Physiol.* 149, 1860–1871. doi: 10.1104/pp.108.133934

Zinn, K. E., Tunc-Ozdemir, M., and Harper, J. F. (2010). Temperature stress and plant sexual reproduction: uncovering the weakest links. *J. Exp. Bot.* 61, 1959–1968. doi: 10.1093/jxb/erq053

Zinselmeier, C., Jeong, B. R., and Boyer, J. S. (1999). Starch and the control of kernel number in maize at low water potentials. *Plant Physiol.* 121, 25–35. doi: 10.1104/pp.121.1.25

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Regulation of leaf hydraulics: from molecular to whole plant levels

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The water status of plant leaves is dependent on both stomatal regulation and water supply from the vasculature to inner tissues. The present review addresses the multiple physiological and mechanistic facets of the latter process. Inner leaf tissues contribute to at least a third of the whole resistance to water flow within the plant. Physiological studies indicated that leaf hydraulic conductance (K_{leaf}) is highly dependent on the anatomy, development and age of the leaf and can vary rapidly in response to physiological or environmental factors such as leaf hydration, light, temperature, or nutrient supply. Differences in venation pattern provide a basis for variations in K_{leaf} during development and between species. On a short time (hour) scale, the hydraulic resistance of the vessels can be influenced by transpiration-induced cavitations, wall collapses, and changes in xylem sap composition. The extravascular compartment includes all living tissues (xylem parenchyma, bundle sheath, and mesophyll) that transport water from xylem vessels to substomatal chambers. Pharmacological inhibition and reverse genetics studies have shown that this compartment involves water channel proteins called aquaporins (AQPs) that facilitate water transport across cell membranes. In many plant species, AQPs are present in all leaf tissues with a preferential expression in the vascular bundles. The various mechanisms that allow adjustment of K_{leaf} to specific environmental conditions include transcriptional regulation of AQPs and changes in their abundance, trafficking, and intrinsic activity. Finally, the hydraulics of inner leaf tissues can have a strong impact on the dynamic responses of leaf water potential and stomata, and as a consequence on plant carbon economy and leaf expansion growth. The manipulation of these functions could help optimize the entire plant performance and its adaptation to extreme conditions over short and long time scales.

Keywords: aquaporin, hydraulic conductance, leaf growth, veins, xylem

INTRODUCTION

The growth of plants is critically dependent on two key physiological processes that occur in leaves: gas exchange through stomata and carbon fixation in the photosynthetic tissues. To operate optimally, these processes require a well-balanced hydration status of the leaf.

The water status of plant leaves is dependent on both stomatal regulation and water supply from the vasculature to inner tissues. The present review addresses the multiple physiological and mechanistic facets of the latter process. Following uptake by root and transport to shoots via vascular tissues, water (xylem sap) is delivered throughout the whole leaf lamina, before evaporating in the substomatal chambers and diffusing through the stomata (Sack and Holbrook, 2006). A small portion of the leaf water flow is used to support expansion growth (Pantin et al., 2012).

Water transport in leaves is therefore mediated through a complex network of hydraulic structures. The organization of this network is dictated by independent structural constraints, for optimizing sap delivery and other leaf functions such as light harvesting (Brodribb and Feild, 2010). Beyond anatomical features, our understanding of most of molecular and genetic mechanisms involved in leaf water transport is incomplete. In particular, the respective contributions of the vessels and the living tissues to water transport as well as the pathways used by water in the latter tissues are extensively studied. Recent advances about the mechanisms that allow the adjustment of leaf hydraulics in response to developmental and environmental factors will also be presented.

LEAF HYDRAULIC CONDUCTANCE: A HIGHLY VARIABLE PARAMETER

LEAF HYDRAULIC CONDUCTANCE

Following its delivery from the stem as xylem sap, liquid water flows through veins, and crosses the xylem parenchyma, bundle sheath, and mesophyll tissues before evaporating in leaf air spaces and substomatal chambers. Thus, water transport in leaves involves two states of water, liquid and gaseous.

The transport of liquid water in inner leaf tissues, which is the object of the present review, is governed by classical flow equations used in plant water relations (Steudle, 1989). These equations tell us that water transport intensity is linearly linked to both the driving force (water potential gradient) between the petiole and the substomatal cavity and the water transport capacity (hydraulic conductance) of the leaf (K_{leaf}; Sack and Holbrook, 2006). K_{leaf} is therefore a key physiological parameter to address the transport of liquid water in the leaf, while excluding the contribution of

stomata to water vapor diffusion. K_{leaf} integrates all water transport paths working in parallel or in series within the inner leaf tissues, each having its own physical characteristics.

TECHNIQUES FOR MEASURING WATER TRANSPORT IN WHOLE LEAVES

Experimentally, K_{leaf} is determined as the ratio of water flow rate through the leaf to the driving force, that is, the water potential difference between the petiole and leaf lamina (ideally, the substomatal chambers). K_{leaf} is usually normalized by leaf area (Sack and Holbrook, 2006). At the whole leaf level, three major techniques have been developed to measure K_{leaf}.

The evaporative flux method (EFM) is the most commonly used. It relies on the relationship that exists, under steady state conditions, between the flux of transpiration across the plant or an excised leaf and the corresponding drop in water potential (Martre et al., 2002; Sack et al., 2002). K_{leaf} is deduced from the ratio of transpiration flow to the difference of water potential between the stem and the leaf. In practice, water potentials are measured in a fully transpiring leaf and in a leaf covered with a bag to locally prevent any transpiration. The latter leaf reports on the stem water potential.

The high pressure method (HPM) requires a flow of solution to be pushed using a pump, from the petiole throughout the leaf (Sack et al., 2002; Tyree et al., 2005). Alternatively, an excised leaf or rosette can be inserted into a pressure chamber whereby a flow of solution is pressed through the stomata and exits the leaf through the hypocotyl section (Postaire et al., 2010). K_{leaf} can be deduced from the flow vs. pressure relationship. It has been argued that stomatal constrictions could dominate the measured K_{leaf}. However, Poiseuille's law indicates that, by contrast to vapor phase transport, the pore apertures must represent a negligible resistance under conditions of liquid flow. These assumptions were supported experimentally in walnut (*Juglans regia*) which leaves showed a marked stomatal closure in response to abscisic acid (ABA), without any alteration in K_{leaf} (Tyree et al., 2005) and in model species *Arabidopsis thaliana* which rosette hydraulic conductivity was increased under darkness, simultaneously to stomatal closure (Postaire et al., 2010).

The vacuum pump method (VPM) represents the third type of K_{leaf} measuring method. In this case, water enters an excised leaf through its sectioned petiole. The leaf blade is carefully maintained at saturating water vapor but subjected to vacuums of different intensities. Thus, water is pulled by suction, in the absence of any vapor pressure deficit and the measured K_{leaf} mostly reflects a liquid phase conductance of inner leaf tissues (Sack et al., 2002).

There are still ongoing discussions about the respective validity of these three types of K_{leaf} measurement methods (Rockwell et al., 2011). For instance, water potential measurements required for the EFM have many pitfalls. However, this method is performed in conditions whereby water evaporates in the leaf airspaces and diffuses from the stomata, and it has been argued that, with regard to other methods, EFM most closely reports on the natural pathway of water in leaves (Sack and Scoffoni, 2012). In contrast, the HPM and VPM may not yield K_{leaf} values that reflect the *in vivo* context, since a flow of water is driven through the leaf at higher hydrostatic pressure gradients than ambient. In addition, during HPM measurements (and perhaps to some degree with the VPM), the leaf or rosette is flooded with a liquid solution and leaf airspaces rapidly become infiltrated. This may create novel pathways for water movement, in addition to those utilized during transpiration. Yet, several comparative studies, including one with six woody angiosperm species, showed that similar K_{leaf} values (with differences around 10%) could be determined by the three methods (Sack et al., 2002). From this, it was inferred that the mesophyll pathway that may be shunted when using the HPM may be of negligible resistance (Sack et al., 2002).

LEAF HYDRAULIC CONDUCTANCE VALUES ACROSS PLANT SPECIES

A comprehensive set of K_{leaf} data has now been collected in the whole plant kingdom. These studies revealed that K_{leaf} is highly variable, by up to 65-fold across plant species (Sack et al., 2005).

These studies also established that, with respect to roots and stems, leaf tissues can represent a substantial part of the inner resistance to whole plant water flow. Within a sample of 34 species, the leaf contributed on average a third of the whole plant resistance (Sack et al., 2003) but in some cases it could represent up to 98% of this resistance (Sack and Holbrook, 2006). Of outstanding interest for the physiologist is also the observation that K_{leaf} can be highly variable and dynamic during plant life. Thus, K_{leaf} depends on the anatomy and developmental stage of the leaf; it can also vary according to plant growing conditions, over a wide range of time scales, from minutes to months.

VARIATION OF LEAF HYDRAULIC CONDUCTANCE IN RESPONSE TO DEVELOPMENTAL AND ENVIRONMENTAL FACTORS

DEVELOPMENT

K_{leaf} shows dynamic changes over the whole leaf lifetime, with patterns specific for each species (Aasamaa et al., 2005; Nardini et al., 2010). Generally, K_{leaf} increases in developing leaves as the vasculature matures. In the weeks or months following its maximum, K_{leaf} begins to decline, by up to 80–90% at abscission (Aasamaa et al., 2005; Brodribb et al., 2005). Some authors have hypothesized that seasonal decline of K_{leaf} is a trigger for leaf senescence (Sack and Holbrook, 2006).

IRRADIANCE

Variations in K_{leaf} due to changes in irradiance have now been reported in numerous plant species. In most cases, K_{leaf} is the lowest at low irradiance (<10 μmol photons m^{-2} s^{-1}) or under darkness (Sack et al., 2002; Nardini and Salleo, 2005; Tyree et al., 2005). In sunflower (*Helianthus annuus*) for instance, K_{leaf} is reduced by 30–40% during the night compared to the day (Nardini and Salleo, 2005). Conversely, K_{leaf} can rapidly increase by several-fold in response to a high irradiance (up to >1000 μmol photons m^{-2} s^{-1}; Sack et al., 2002; Lo Gullo et al., 2005). For instance, K_{leaf} was increased in the 30 min following a transition to high light in 6 out of 11 tropical plant species (Tyree et al., 2005). Light quality has also an important impact on leaf hydraulic properties. In silver birch (*Betula pendula*; Sellin et al., 2011) and cucumber (*Cucumis sativus*) leaves (Savvides et al., 2012), K_{leaf} was the highest under blue light, intermediate under white light, and the lowest under red light. It is of note that the K_{leaf} of *Arabidopsis* is also regulated

by the light regime, but unlike the majority of species studied, it was increased by about 40% during the night and by twofold when night was extended by 5–15 h (Postaire et al., 2010).

More generally, K_{leaf} follows diurnal and seasonal rhythms. For sunflower and some tree species, K_{leaf} increased by up to two to threefold over a few hours from morning to midday and then declined by evening (Lo Gullo et al., 2005; Cochard et al., 2007). When sunflower plants were kept in the dark for several days, K_{leaf} continued to oscillate in phase with the subjective light period, indicating that these changes were driven by the circadian clock (Nardini and Salleo, 2005).

DROUGHT STRESS

Leaves are able to sense and respond to various types of water shortage (Sack and Holbrook, 2006). When *Arabidopsis* plants were exposed to low air humidity (implying higher transpiration) a concomitant increase in K_{leaf} and whole plant hydraulic conductance was observed (Levin et al., 2007). ABA is also a central mediator of plant response to drought stress. Inhibiting effects of ABA on inner leaf water transport (K_{leaf}) were recently revealed in *Arabidopsis* (Shatil-Cohen et al., 2011). In this study, ABA was fed to excised leaves through the xylem via transpiration. Pantin et al. (2013) confirmed these effects and showed that xylem-fed ABA decreased K_{leaf} and stomatal conductance (g_s) in mutants that are known to be insensitive to ABA-induced stomatal closure. This suggested that the stomatal regulation was mediated via a hydraulic feedback in a tissue upstream of the stomata (Pantin et al., 2013).

INTERACTION BETWEEN FACTORS ACTING ON LEAF HYDRAULIC CONDUCTANCE

Although most studies have addressed the effects of individual factors on K_{leaf}, an integrated view of the dynamics and combined impacts of irradiance, leaf water status and development on K_{leaf} is now critically needed. This question was recently investigated in sunflower and three shrub species (Guyot et al., 2012). In each case, the amplitude of K_{leaf} response to light or leaf dehydration was positively correlated to the intensity of the other parameter. These properties may allow optimal adjustment of the leaf water status under contrasting conditions when light tends to enhance transpiration whereas soil water availability is declining. These few examples illustrate the diversity of physiological contexts leading to changes in K_{leaf}. The following sections address the variety of molecular and cellular mechanisms involved and the physiological significance of these regulations.

VASCULAR WATER TRANSPORT

CONTRIBUTION OF THE VASCULAR PATHWAY TO LEAF HYDRAULIC CONDUCTANCE

The vascular pathway is composed of a highly structured network of differentiated (non-living) vessels that deliver xylem sap through the entire leaf, close to the evaporation sites. The minimization of transport distances out of the vascular pathway is one key feature of the hydraulic performance of leaves (Sack and Holbrook, 2006). In most dicots, venation is constructed according to a hierarchical order: midvein, second- and third-order veins, and finally minor veins that confer the reticulate pattern (**Figure 1A**). It is generally assumed that, whatever the leaf vascular anatomy, the bulk of transpired water follows the path of lesser resistance down the vein network, from midrib to minor veins before exiting the vessels (Sack and Holbrook, 2006). This means that water subsequently follows the vascular and extravascular paths.

The respective contributions of these two paths to K_{leaf} and to its variations have been the object of numerous studies. Models based on electrical analogies and using Poiseuille's law have been developed to calculate the hydraulic conductance of leaf xylem networks (Lewis and Boose, 1995). They demonstrated the importance of hierarchy in the vein network to optimize water transport (Cochard et al., 2004; McKown et al., 2010). One first method to determine the contribution of veins to the leaf hydraulic resistance (R_{leaf}, the inverse of K_{leaf}) is to cut an increasing number of minor veins. The measured R_{leaf} progressively decreases and converges toward a stable value, the supposed vascular resistance (Sack et al., 2004; Nardini and Salleo, 2005). A second method consists in disrupting the living structures of the leaf by freezing or boiling the entire organ. The measured R_{leaf} is then reduced to its vascular component (Cochard et al., 2004) provided that the treatments do not alter the xylem vessel diameter or the extensibility of the walls. All these studies have revealed that the hydraulic resistances of the vascular and extravascular compartments are of the same order of magnitude. Either one may prevail, depending on species or environmental factors (Zwieniecki et al., 2002; Sack et al., 2004; Nardini and Salleo, 2005).

VARIATIONS BETWEEN SPECIES

Leaf vascular anatomy is highly variable across species with respect to vein arrangement and density. The number, size, and geometry of the vascular bundles in the veins and of the xylem conduits within the bundles are also very diverse (Roth-Nebelsick et al., 2001). Yet, common principles of organization can be found, such as a global scaling between leaf size and vein characteristics. In particular, larger leaves have major veins of larger diameter, but lower length per leaf area, whereas minor vein traits are independent of leaf size (Sack et al., 2012; Sack and Scoffoni, 2013).

This great anatomic variability could explain to a large extent the dramatic differences in K_{leaf} observed between species. Several main trends relative to measured K_{leaf} variations have been validated through modeling (Cochard et al., 2007; McKown et al., 2010). Firstly, the conductance of the main veins appeared as a major limiting factor of K_{leaf}. By contrast, the arrangement and density of these veins had a marginal impact on K_{leaf} (Sack and Frole, 2006) and would rather contribute to a uniform distribution of water across the lamina (Roth-Nebelsick et al., 2001; Zwieniecki et al., 2002) and avoid cavitation (Sack and Holbrook, 2006). Secondly, the K_{leaf} of plants with a higher minor vein density tended to be greater. This is not due to an increase in conductance of the xylem system *per se* (Cochard et al., 2004), but rather to an increase in the surface area for exchange of xylem sap with surrounding mesophyll and reduced distances in extravascular pathway (Roth-Nebelsick et al., 2001; Sack and Frole, 2006). A high vein density also favors water potential equilibration across the leaf and prevents the damage or blockage of higher-order veins (Sack and Scoffoni, 2013).

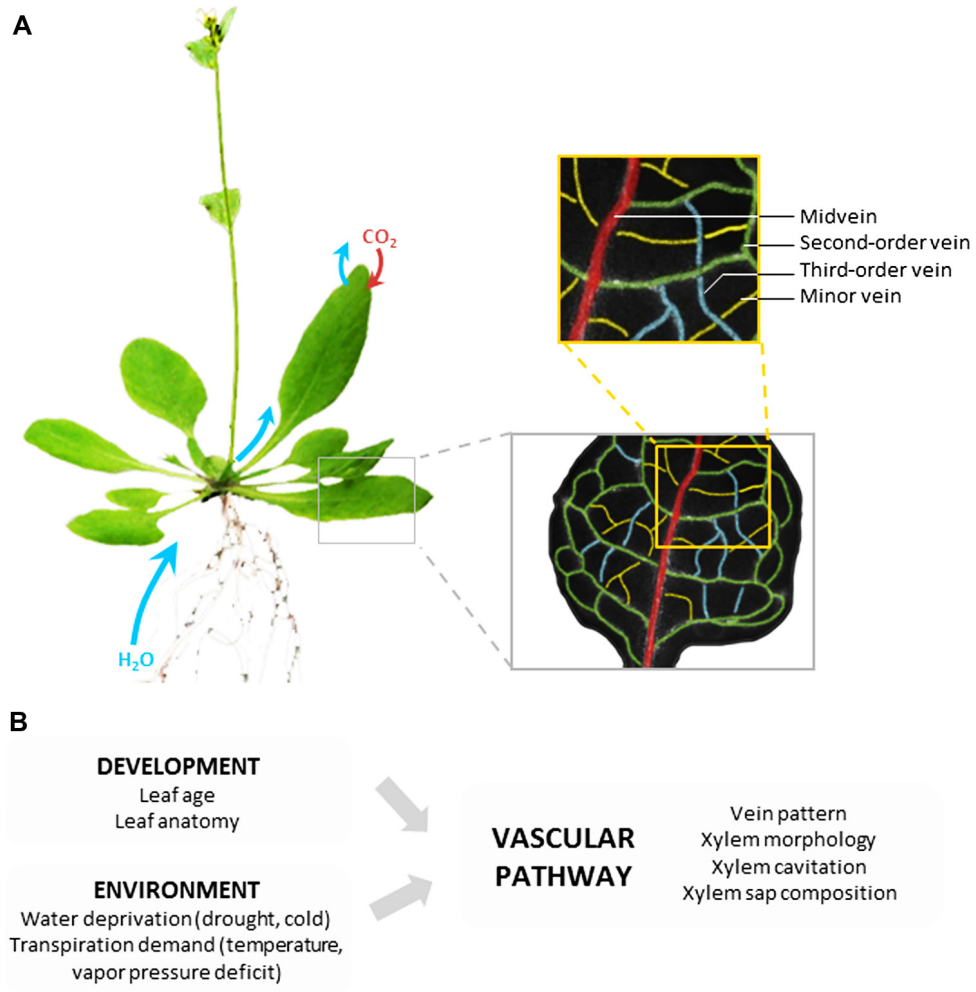

FIGURE 1 | Leaf vascular pathway and regulation of water transport. **(A)** Based on the example of *Arabidopsis thaliana*, the figure shows that following uptake by roots and transfer to shoots, water is delivered throughout the whole leaf lamina by a highly organized network of veins. The non-living vessels form the leaf vascular pathway which is constructed according to a hierarchical order with midvein, second-order veins, third-order veins, and minor veins. The midvein runs from the petiole to the leaf apex, with second-order veins branching at regular intervals, and third-order veins branching on the latter. **(B)** The hydraulic resistance of the vascular pathway can be influenced by various developmental and environmental factors acting on the venation pattern or the indicated xylem properties.

THE CONSTRUCTION COST OF VASCULAR PATHWAYS

The development of a dense vein network represents a massive investment for the plant because lignified tissues are net carbon sinks that do not directly contribute to photosynthesis (Pantin et al., 2012). However, maximum net assimilation rate of photosynthesis depends on the capacity of the leaf vascular system to supply water to photosynthesizing mesophyll cells (Brodribb et al., 2007). Hydraulic modeling of leaves revealed that the conductivity and density profiles of veins of various orders contribute to optimizing the hydraulic efficiency of the xylem network. A high vein density only becomes economically viable compared to the photosynthetic costs when it is supported by a highly conductive low order venation. A high vein density limits the distance of photosynthate and water transport between veins, photosynthesizing mesophyll cells, and evaporative surfaces of the leaf (Amiard et al., 2005; Brodribb et al., 2007; McKown et al., 2010). Hence, the hydraulic properties of the leaf tissue play a fundamental role in linking leaf construction with photosynthetic capacity.

ENVIRONMENTAL EFFECTS

It is of note that, beyond developmental factors, the functioning and hydraulic resistance of the vascular pathway depends on the plant growth conditions (Brodribb et al., 2010). The combined use of a xylem pressure probe and a Scholander–Hammel pressure bomb in intact maize (*Zea mays*) plants was used to demonstrate that leaf xylem pressure can change rapidly and reversibly with environmental modifications, such as light intensity or soil water potential (Wei et al., 1999). One striking consequence is water stress-induced xylem cavitations that result in marked reductions in K_{leaf} (Bucci et al., 2003; Nardini et al., 2003; Johnson et al., 2009). However, decrease of K_{leaf} in dehydrating pine needles

(Cochard et al., 2004) appeared to be due to a collapse of tracheids. On the longer term, water shortage can interfere with leaf growth and xylem differentiation. In sunflower for instance, K_{leaf} was decreased in response to 20-day-long moderate or severe water stresses due to narrower xylem conduits (Nardini and Salleo, 2005). During winter, freeze–thaw cycles in vessels of woody plants can also result in xylem vessel embolism and/or wall collapse and therefore induce a significant decrease in K_{leaf} (Ameglio et al., 2001). Hence, different plant species may exhibit contrasting vulnerability to water stress- or winter-induced embolism, depending on the anatomy of their vessels.

The xylem sap composition, and in particular its potassium concentration, can interfere with the wall permeability of tracheids (Zwieniecki et al., 2001). These effects may be due to a shrinking and swelling of the pectin hydrogel forming the inter-vessel pit membranes. This mechanism which impacts K_{leaf} has been invoked to explain the effects of light on stem hydraulics in laurel and silver birch (*Betula pendula*; Nardini et al., 2010; Sellin et al., 2010).

In conclusion, the vascular compartment of leaves allows a broad range of hydraulic configurations between species, during development or in response to environment fluctuations (**Figure 1B**). As explained in the next sections, the extravascular structures can provide complementary means for rapid and reversible regulations of K_{leaf} (Sack and Holbrook, 2006).

THE EXTRAVASCULAR COMPARTMENT

WATER PATHWAYS INSIDE THE EXTRAVASCULAR COMPARTMENT

The extravascular compartment includes all living tissues that transport water from xylem vessels to substomatal chambers. Following its exit from xylem conduits, water flows through xylem parenchyma cells and enters the bundle sheath made up of parenchymatous cells wrapped around the veins (Leegood, 2008). Water then crosses bundle sheath extensions or the mesophyll to reach the epidermis and evaporations sites, respectively. The location and surface area of the latter sites may vary according to leaf anatomy, some species having huge leaf internal airspaces (Brodribb and Feild, 2010; **Figure 2A**). Recently, a shift has been made from the simple idea that leaves can be reduced to a single pool of evaporating water to a more complex leaf representation with well-organized water pools separated by hydraulic resistances (Zwieniecki et al., 2007).

It is classically assumed that water can follow different paths to flow across living tissues, from cell-to-cell, through cell membranes (transcellular path) and plasmodesmata (symplastic path), or through the continuity of walls (apoplastic path; Steudle and Peterson, 1998). The relative contribution of these different paths in leaves is currently unclear and could vary according to species, leaf developmental stage (Voicu and Zwiazek, 2010), or physiological conditions (Sack et al., 2004; Nardini and Salleo, 2005; Cochard et al., 2007; Ye et al., 2008). Tissue anatomy can provide preliminary hints at these questions. Mesophyll tissues often have a low cell packing and are largely composed of airspaces. This, and experiments whereby apoplastic transport was traced using dyes such as 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), have suggested that apoplastic water movement predominates during transpiration (Sack and Holbrook, 2006; Voicu et al., 2008, 2009). Water may cross cell membranes only for cell water homeostasis, during rehydration and expansion growth (Heinen et al., 2009). In contrast, the vascular bundles show physically tight cell layers (**Figure 2A**). In addition, recent work indicated that bundle sheath cells may have suberin lamellae and/or apoplastic barriers on radial walls, thereby decreasing the apoplastic flow of water (Lersten and Curtis, 1997). Thus, transcellular water flow may be critical at this site.

THE DYNAMICS OF LEAF CELL WATER PERMEABILITY IN RESPONSE TO DEVELOPMENTAL AND ENVIRONMENTAL FACTORS

Several techniques have been developed to measure the water permeability of leaf cells and therefore dissect the functional behavior of the extravascular pathway. The cell pressure probe technique which gives access to cell water relation parameters in intact plant tissues has been applied to several cell types including the stomata, epidermis, mesophyll (Franks, 2003), and midrib parenchyma (Kim and Steudle, 2007, 2009). Since this technique is not applicable to small sized or deeply embedded cells, cell water permeability can also be characterized by means of osmotic swelling assays in protoplasts. The protoplasts are isolated according to their morphology or to cell-specific expression of fluorescent reporter proteins. This approach has been developed firstly in mesophyll protoplasts of various plant species (Ramahaleo et al., 1999; Morillon and Chrispeels, 2001; Martre et al., 2002) and more recently in protoplasts from *Arabidopsis* bundle sheath (Shatil-Cohen et al., 2011) and xylem parenchyma (Prado et al., 2013). In general, the water permeability of protoplasts is lower than in intact cells (Moshelion et al., 2004; Chaumont et al., 2005; Hachez et al., 2006, 2008; Volkov et al., 2007).

These techniques have first revealed that cell water permeability can vary according to leaf developmental stage (**Figure 2B**). In barley (*Hordeum vulgare*) and maize leaves, the water permeability of protoplasts isolated from the zones of emergence, elongation, and maturation was the highest in the former zone (Volkov et al., 2007; Hachez et al., 2008). A high cell water permeability may be beneficial during tissue expansion.

Measurements in individual leaf cells have also indicated that changes in K_{leaf} induced by environmental factors on the short-term may be mediated through changes in cell membrane water permeability (**Figure 2B**). For instance, the water permeability of individual parenchyma cells, as measured with a cell pressure probe in the midrib of maize leaves, was increased by up to three-fold at low light intensities (Kim and Steudle, 2007). Other studies using protoplast swelling assays showed that, in maize, the leaf cell water permeability was the highest during the early hours of the day (Hachez et al., 2008). A similar approach revealed that diurnal leaf movements in rain tree (*Samanea saman*) and tobacco were linked to regulation of cell water transport in pulvini and petiole, respectively (Moshelion et al., 2002; Siefritz et al., 2004). The transpiration demand can also impact leaf cell water permeability. In *Arabidopsis* plants grown under various transpiring regimes or ABA treatments (Morillon and Chrispeels, 2001), an inverse relationship was found between mesophyll protoplast water permeability and the rate of plant transpiration which, however, could not be attributed to a direct action of ABA on the mesophyll. Bundle sheath cells seem to have, by contrast, a specific responsiveness

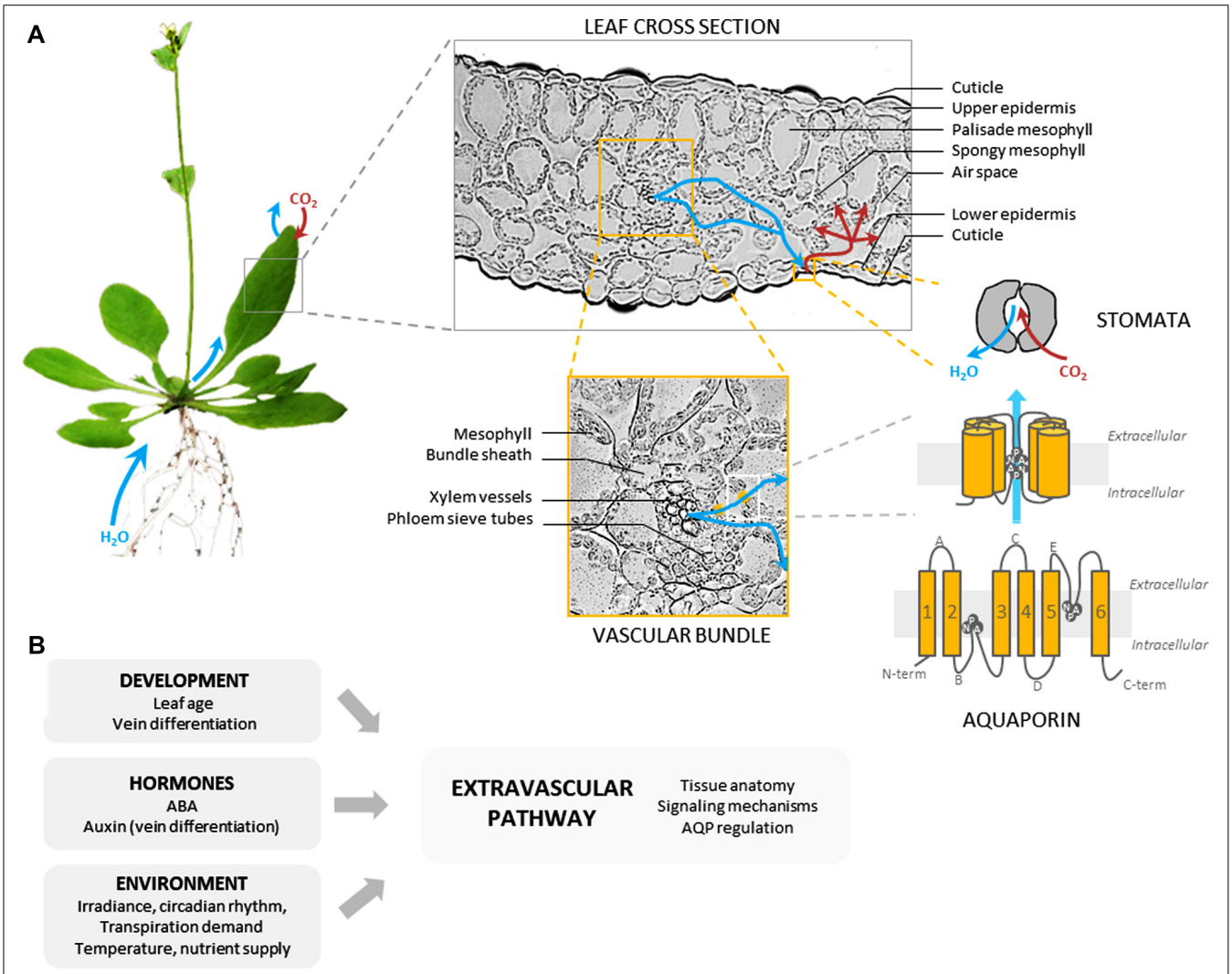

FIGURE 2 | Leaf extravascular pathway and regulation of water transport. (A) The figure shows, using the *Arabidopsis* leaf as an example, the various components of the extravascular pathway, from whole organ to molecular levels. The pathway followed by water between xylem vessels and substomatal chambers is not entirely understood. Whereas the role of xylem parenchyma and bundle sheath is emerging (see text), the contribution of the mesophyll may depend on leaf anatomy. Water transport across living cells is mediated in part by water channel proteins called aquaporins (AQPs) formed by six α-helical transmembrane domains linked by five loops (A–E), and N- and C-terminal ends localized in the cytosol. Two specific, highly conserved structural motifs (NPA) are located in the pore and contribute to AQP selectivity. AQPs are expressed in all leaf living cells but preferentially in veins. **(B)** Various developmental and environmental factors act on the indicated components of the extravascular pathway to alter its hydraulic properties. AQP regulation occurs at various levels including gene expression, AQP trafficking and gating (see text).

to ABA which could explain the down-regulating effects of this hormone on K_{leaf} (Shatil-Cohen et al., 2011).

HYDRAULIC LIMITATIONS IN THE EXTRAVASCULAR COMPARTMENT

The nature of the living cells that, within the leaf, oppose the major hydraulic resistance to the transpiration flow is still under debate (Cochard et al., 2004; Sack et al., 2004; Nardini and Salleo, 2005; Voicu et al., 2008). One recent approach made use of a non-invasive leaf pressure probe in *Arabidopsis* leaves (Ache et al., 2010). This new technique indicated that mesophyll cell turgor was markedly reduced at high transpiration rate, suggesting that an upstream structure, possibly the bundle sheath, was hydraulically limiting. In support for this, Shatil-Cohen et al. (2011) observed a correlation between the effects of ABA on K_{leaf} and the water permeability of protoplasts from the bundle sheath but not from mesophyll. This correlative approach was recently extended by Prado et al. (2013) who considered a larger set of vein protoplasts in *Arabidopsis* leaves. The data indicated that xylem parenchyma, in addition to bundle sheath, may be limiting during K_{leaf} regulation by light. A hydraulic limitation due to the xylem parenchyma was already suggested in maize leaf (Tang and Boyer, 2002). We note, however, that these conclusions may not apply to tobacco which showed no correlation between the hydraulic conductivities of whole leaves and bundle sheath cells (Lee et al., 2009). In addition, bundle sheath extensions which in some species link the bundle sheath to the epidermis and separate the leaf into chambers

may influence the dynamics of K_{leaf} in response to irradiance and leaf water status (Sack and Scoffoni, 2013).

Altogether, water transport measurements in leaf cells have led to the realization that many of developmentally and environmentally induced variations of K_{leaf} may be explained through regulations of cell membrane water transport. Aquaporin (AQP) water channels are membrane proteins that facilitate the exchange of water across cell membranes and can be responsible for up to 95% of the water permeability of plant plasma membranes (Maurel et al., 2008). This explains the intensive research recently developed on the function and regulation of AQPs in leaves.

AQUAPORINS IN LEAVES: TISSUE-SPECIFICITY AND PUTATIVE ROLES

THE AQP FAMILY OF WATER CHANNEL PROTEINS

AQPs have a characteristically conserved structure with monomers (23–31 kDa) comprising six α-helical transmembrane domains linked by five loops (A–E) and N- and C-terminal ends localized in the cytosol (**Figure 2A**). AQPs assemble as tetramers, each monomer forming an individual transmembrane pore (Wang and Tajkhorshid, 2007). Plant AQPs show a great diversity, with >30 isoforms in higher plant species. They fall into at least four major homology subgroups that somehow reflect specific subcellular localizations (Maurel et al., 2008). For instance, the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) represent the most abundant AQPs in the plasma membrane and in the tonoplast, respectively. The great diversity of plant AQPs also reflects a broad range of transport specificities (Tyerman et al., 2002). In addition to water, some AQP isoforms can transport non-polar solutes such as metalloids (Bienert et al., 2008), gases (Uehlein et al., 2003), or reactive oxygen species (ROS; Bienert et al., 2007; Dynowski et al., 2008), suggesting multiple functions, in water and nutrient transport, and cell signaling.

TISSUE-SPECIFIC EXPRESSION OF AQPs AND PUTATIVE ROLES

Expression profiling of the *AQP* gene family in several plant species has indicated that leaves are equipped with multiple AQP isoforms. By contrast to what was observed in pollen or seeds, no AQP transcript was strictly specific for leaves. In the *Arabidopsis* leaf, two *TIP* (*AtTIP1;2* and *AtTIP2;1*) and three *PIP* (*AtPIP1;2*, *AtPIP2;1*, and *AtPIP2;6*) genes are strongly expressed and *AtPIP2;6* shows preferential expression in this organ (Jang et al., 2004; **Figure 3**). Quantitative proteomics of plasma membranes purified from *Arabidopsis* leaves confirmed this pattern and showed that *At*PIP1;2, *At*PIP2;1, and *At*PIP2;7 were the most abundant among the nine PIPs isoforms detected (Monneuse et al., 2011).

Beyond these global studies, the marked cell-specific expression patterns of some isoforms can provide interesting hints at a variety of AQP functions in the leaf. In tobacco for instance, strong expression of a PIP1 homolog, *Nt*AQP1, was observed in spongy parenchyma cells of mesophyll, with the highest concentration around substomatal cavities (Otto and Kaldenhoff, 2000). AQPs may fulfill multiple roles in the mesophyll: transcellular water transport during transpiration, as suggested for *Nt*AQP1, but also cell osmotic adjustment under varying water demand, or CO_2 transport (Otto and Kaldenhoff, 2000).

Yet, a preferential expression of AQPs in the vascular bundles was observed in many plant species, suggesting a special role for AQPs in delivering water from the vessels to the mesophyll (Kaldenhoff et al., 2008). In particular, bundle sheath cells were shown to have high PIP and TIP expression levels in rapeseed (*Brassica napus*; Frangne et al., 2001), *Arabidopsis* (Kaldenhoff et al., 1995; Prado et al., 2013), ice plant (*Mesembryanthemum crystallinum*; Kirch et al., 2000), Norway spruce (*Picea abies*; Oliviusson et al., 2001), maize (Hachez et al., 2008), and rice (*Oryza sativa*; Sakurai et al., 2008). This expression pattern is consistent with the observation that the bundle sheath is formed of highly compacted cells, with sometimes lignified or suberized cell walls (see Water Pathways Inside the Extravascular Compartment). Strong expression of AQPs in the xylem parenchyma has also been described in several species (Barrieu et al., 1998; Otto and Kaldenhoff, 2000; Sakr et al., 2003; Hachez et al., 2008; Prado et al., 2013). This site of expression may be crucial for radial cell-to-cell water movement during exit from the xylem vessels (Prado et al., 2013) and for osmotically driven water loading in xylem vessels during embolism refilling (Sakr et al., 2003; Secchi and Zwieniecki, 2010). AQPs were also found to be abundant in phloem companion cells (Kirch et al., 2000; Fraysse et al., 2005) suggesting a role in phloem sap loading and in maintaining vascular tissue functions under drought stress (Montalvo-Hernandez et al., 2008). Finally, AQPs are expressed in epidermis (Cui et al., 2008), trichomes, stomata (Heinen et al., 2009), and dividing cells (Barrieu et al., 1998) where their role still needs to be established.

This survey should not give a static view of AQP expression, which is constantly adjusted during leaf development. In maize and barley leaves for instance, some isoforms were highly expressed in young, elongating leaf tissues whereas others were preferentially expressed in fully developed, matured tissues (Wei et al., 2007; Hachez et al., 2008; Besse et al., 2011; Yue et al., 2012).

INVOLVEMENT OF AQPs IN LEAF HYDRAULICS: PHARMACOLOGICAL AND GENETIC EVIDENCES

The contribution of AQPs to leaf water transport was first demonstrated using pharmacological inhibition. Treatment of mesophyll and bundle sheath protoplasts with mercury, which blocks AQPs through oxidation of Cys residues, resulted in a fivefold reduction in cell water permeability (Kaldenhoff et al., 1998; Shatil-Cohen et al., 2011). At the whole leaf level, mercury treatment decreased K_{leaf} by 33% in sunflower (Nardini and Salleo, 2005) and by around 40% in six temperate deciduous trees (Aasamaa et al., 2005). Although it is also rather unspecific and toxic, azide, which induces cell acidosis and a pH-dependent closure of PIPs (Tournaire-Roux et al., 2003), was used in *Arabidopsis* as an independent type of AQP blocker. The similar inhibiting effects of mercury and azide supported the idea that, in this species, PIPs truly contribute to the enhancement of rosette hydraulic conductivity under darkness (Postaire et al., 2010).

Given the lack of specific inhibitors, genetic approaches provide a more reliable approach for studying the physiological function of plant AQPs. *Arabidopsis* plants expressing *AtPIP1;2* or *AtPIP2;3* antisense transgenes, individually or in combination, showed in parallel to a reduced expression of PIP1s and/or PIP2s, a 5- to 30-fold reduction in water permeability of isolated mesophyll

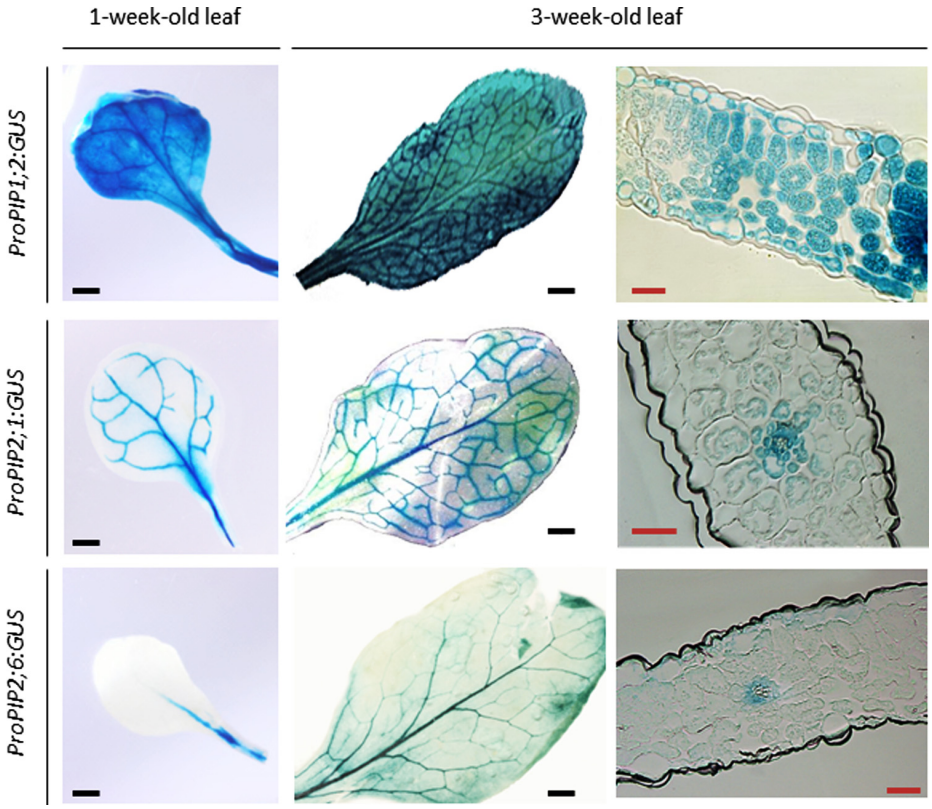

FIGURE 3 | Expression pattern of three highly expressed *PIP* genes during leaf development in *Arabidopsis*. The figure shows expression in transgenic plants of chimeric genes expressing a β-*glucuronidase* gene (*GUS*) under the control of *PIP* promoter sequences (*ProPIP*). The three indicated AQP isoforms (*At*PIP1;2, *At*PIP2;1, *At*PIP2;6) contribute to rosette hydraulic conductivity (Prado et al., 2013). Cross-sections show intense staining in the veins of each type of transgenic plants (black scale bar = 2.5 mm, red scale bar = 0.1 mm).

protoplasts (Kaldenhoff et al., 1998; Martre et al., 2002). The antisense lines also showed a leaf water potential and a K_{leaf} significantly lower than in control plants, only under water limiting conditions. The differences were stronger during re-watering, suggesting that AQP-mediated water transport was directly involved in leaf tissue rehydration (Martre et al., 2002). In tobacco, the phenotype of an antisense *NtAQP1* line suggested that this AQP is involved in the differential expansion growth of the upper and lower surfaces of the petiole during leaf unfolding (Siefritz et al., 2004). The contribution of individual AQPs to water leaf transport was thoroughly dissected in *Arabidopsis*. Plant lines carrying an individual T-DNA insertion in three out of four highly expressed *PIP* genes (*AtPIP1;2, AtPIP2;1, AtPIP2;6)* displayed, when grown in the dark, reduction in K_{leaf} by approximately 30%, similar to the reduction displayed by a corresponding triple *pip* mutants (Prado et al., 2013). Another study using a deuterium tracer method to assess water relocation in *Arabidopsis* showed that K_{leaf} was significantly reduced by about 20% in *pip2;1* and *pip2;2* knock-out plants (Da Ines et al., 2010).

AQUAPORINS IN LEAVES: MODES OF REGULATION

RESPONSE TO LIGHT AND CIRCADIAN RHYTHM

Understanding the molecular and cellular bases of AQP regulation in leaves, and therefore the modes of K_{leaf} regulation in response to developmental or environmental cues, represents an important focus in current research. Because of the dominating role of light and circadian rhythms in regulating K_{leaf}, most of recent studies have been performed in this context. Combined HPM and quantitative RT-PCR analyses in detached walnut leaves revealed a positive correlation between the increase in K_{leaf} under high irradiance and the transcript abundance of two *PIPs*, *JrPIP2;1* and *JrPIP2;2* (Cochard et al., 2007). The light-dependent stimulation of K_{leaf} in European beech (*Fagus sylvatica*) and pedunculate oak (*Quercus robur*) was also associated to enhanced expression of *PIP1* genes (Baaziz et al., 2012). Diurnal oscillations in expression of *NtAQP1* in tobacco leaf petioles (Siefritz et al., 2004), *SsAQP2* in motor cells of *Samanea saman* leaves (Moshelion et al., 2002), and most of *ZmPIP* genes in maize leaves (Hachez et al., 2008) were correlated to changes in water permeability of corresponding protoplasts. However, light-dependent K_{leaf} was not associated to any AQP transcriptional control in certain species such as bur oak (Voicu et al., 2009). Quantitative proteomic analysis in the *Arabidopsis* rosette showed that the abundance of each of the nine detected PIP isoforms was perfectly stable regardless the light regime (Prado et al., 2013). In contrast, the diphosphorylation of *At*PIP2;1 at two C-terminal sites (Ser280 and Ser283) was enhanced by twofold under the same conditions. Whereas the rosette hydraulic conductivity of a *pip2;1* knock-out mutant had

lost any responsiveness to the light regime, expression in the same background of phosphomimetic and phosphorylation deficient forms of *At*PIP2;1 demonstrated that phosphorylation at Ser280 and Ser283 was necessary for K_{leaf} enhancement under darkness (Prado et al., 2013).

WATER STRESS

Plants can undergo water stress in response to numerous environmental constraints such as drought, low atmospheric humidity, salinity, or cold. Studies trying to relate physiological responses to water stress with expression profile of AQPs have led to contrasting results depending on the time course and intensity of water stress (Tyerman et al., 2002; Galmés et al., 2007). Some studies have shown, however, that water stress can coordinately alter AQP expression and activity in the leaf. In grapevine (*Vitis vinifera*) under reduced irrigation for instance, the K_{leaf} was decreased by about 30% together with the expression of *VvTIP2;1* and *VvPIP2;1* (Pou et al., 2013). A low humidity treatment also induced a coordinated up-regulation of many *PIP* and *TIP* genes in rice leaves (Kuwagata et al., 2012). Enhanced expression of some AQPs may also support a role in embolism refilling. For instance, *JrPIP2* which was highly expressed in vessel-associated cells of walnut leaves during the winter period (Sakr et al., 2003).

Proteomic approaches have provided complimentary insights into the mode of AQP regulation under drought. A label-free quantitative shotgun approach in rice leaves under moderate or extreme drought or re-watering conditions showed that most of the nine AQPs identified were responsive to drought, with six decreasing rapidly during plant re-watering (Mirzaei et al., 2012). Phosphoproteomic analyses of *Arabidopsis* seedlings indicated that the C-terminal phosphorylation of *At*PIP2;1 decreased after 30 min of an ABA treatment (Kline et al., 2010). This observation is consistent with the down-regulating effects of ABA on *Arabidopsis* K_{leaf} through a mechanism that involves bundle sheath cells (Shatil-Cohen et al., 2011; Pantin et al., 2013). Thus, similar to what was described in leaves under changing light (Prado et al., 2013), altered phosphorylation of AQPs in veins may act on their trafficking and gating (Törnroth-Horsefield et al., 2006; Prak et al., 2008; Eto et al., 2010) to adjust leaf hydraulics during plant response to drought. The decreased phosphorylation of spinach *So*PIP2;1 following a hyperosmotic treatment in leaf fragments (Johansson et al., 1996) was initially interpreted in the context of leaf cell turgor regulation, whereby an enhanced activity (phosphorylation) of *So*PIP2;1 would favor water influx under fully hydrated conditions. It could also correspond to a water stress-dependent regulation of K_{leaf}.

SIGNALING MECHANISMS ACTING UPSTREAM OF AQP REGULATION

The signaling mechanisms that act upstream of leaf AQP regulation now represent a critical challenge for future research. They likely involve ROS and calcium (Ca^{2+}), which both display specific signatures during leaf response to environmental or hormonal stimuli.

Hydrogen peroxide (H_2O_2) is now recognized as a potent regulator of plant AQPs. H_2O_2 perfusion via the petiole decreased by up to 30-fold the water permeability of epidermal and parenchyma cells, in wandering jew (*Tradescantia fluminensis*; Ye et al., 2008) and maize (Kim and Steudle, 2009) leaves, respectively. A ROS-dependent down-regulation of AQPs has also been invoked to explain the inhibition at high light intensities of the hydraulic conductivity of parenchyma cells, in the midrib tissues of maize leaves (Kim and Steudle, 2009). The mode of action of ROS on water transport is still debated. Hydroxyl radicals produced from exogenously supplied H_2O_2 may act on AQP gating by direct oxidation (Henzler et al., 2004). Such effects were not observed in *Arabidopsis* whereby H_2O_2 triggers a cell signaling cascade ultimately leading to PIP down-regulation, through altered phosphorylation and/or cellular internalization (Boursiac et al., 2008; Prak et al., 2008).

Ca^{2+} plays key structural and signaling roles in plants. It can directly inhibit PIP activity *in vitro* (Gerbeau et al., 2002; Alleva et al., 2006; Verdoucq et al., 2008) by a molecular mechanism that involves Ca^{2+} binding to the cytosolic side of the AQP to stabilize its closed conformation (Hedfalk et al., 2006; Törnroth-Horsefield et al., 2006). This effect has not yet been related to any physiological process in the plant. Plant AQPs can also undergo Ca^{2+}-dependent phosphorylation, which in turn increases their water channel activity. For instance, *in vitro* phosphorylation of spinach leaf PM28A (*So*PIP2;1) was mediated by a plasma membrane-associated protein kinase that was strictly dependent on submicromolar concentrations of Ca^{2+} (Johansson et al., 1996; Sjövall-Larsen et al., 2006). This and other protein kinases acting on leaf AQPs still await biochemical and molecular characterization. An integrative model that links the water flow pathways and Ca^{2+} distribution in leaves was recently proposed (Gilliham et al., 2011). According to this model, the delivery of apoplastic Ca^{2+} and its storage could determine most of hydraulic regulations involving leaf AQPs.

INTEGRATION AND MANIPULATION OF LEAF HYDRAULICS

LEAF HYDRAULIC CONDUCTANCE AND WATER STATUS

Because the leaf water status is at the cross-road of fundamental physiological functions including carbon fixation and growth, its manipulation or genetic improvement could help optimize the entire plant performance, including yield and adaptation to environmental constraints, over short and long time scales. However, several important principles first need to be emphasized to understand the integrative aspects of plant leaf hydraulics and the potential and possible pitfalls of its manipulation.

The present review addressed plant leaf hydraulics, essentially by looking at the multiple facets of K_{leaf}. It is of note that, in plants under transpiring conditions, the dominating resistance for water transport across the plant does not operate in inner leaf tissues but on vapor diffusion, through stomata and at the leaf surface. Thus, the direct impact of K_{leaf} on the intensity of the leaf transpiration may be marginal. The physiological importance of K_{leaf} should not be underestimated, however, since under a fixed transpiration regime, K_{leaf} strongly impacts on the hydration status of the inner leaf tissues (Tsuda and Tyree, 2000). As explained below, leaf hydraulics has a great significance for growth, due to crucial links between this process and leaf water potential. Water potential maintenance in inner leaf tissues is also linked to hydraulic conductance of vessels and stomata and, as a result, interferes with the transpiration flow. For instance, stimuli such as light that enhance K_{leaf} actually promote water supply to the inner leaf

tissues to prevent an excessive drop in water potential throughout the transpiring leaf (Tsuda and Tyree, 2000). This may help reduce tensions and avoid cavitations in xylem vessels. Conversely, a hydraulic limitation in veins, which can typically be enhanced by ABA-dependent down-regulation of AQPs in these territories, can result in a hydraulic signal to promote stomatal closure in plants under water stress (Pantin et al., 2013). This example emphasizes the fundamental interplay that exists between leaf water potential, K_{leaf} and g_s.

AQPs AND HYDRAULIC CONTROL OF LEAF GROWTH

While most of the water absorbed by the plant is lost by transpiration, a minor fraction is retained for supporting leaf growth (Pantin et al., 2012). Leaf expansion growth primarily results from a fine interplay between cell wall relaxation and cell water potential, which both determine the rate of water inflow (Cosgrove, 1987). It is therefore highly sensitive to the leaf water status and has to be protected from environmental disturbances.

The finding of growth-induced water potential gradients (Fricke, 2002; Tang and Boyer, 2002) provided the first direct evidence that leaf growth can be hydraulically limited. This idea is also supported by enhanced function of AQPs in expanding tissues. In cereal leaves for instance, cell water permeability was higher in the elongation zone than in the emerged non-growing zone (Volkov et al., 2007; Hachez et al., 2008). Preferential expression of AQP isoforms in leaf expanding tissues was described in several plant species (Wei et al., 2007; Hachez et al., 2008). This pattern was not restricted to plasma membrane AQPs since expression of *AtTIP1;1* was associated with cell enlargement in *Arabidopsis* leaves (Ludevid et al., 1992) and enhanced by the growth-promoting hormone gibberellic acid (GA3; Phillips and Huttly, 1994). Vacuolar AQPs may favor the differentiation of a large central vacuole that is characteristic of fully elongated cells (Ludevid et al., 1992). Whole plant measurements have also provided evidence for hydraulic limitation of leaf growth. In *Arabidopsis*, it occurs during leaf ontogeny, with leaf growth becoming slower during the day than at night (for a review, see Pantin et al., 2012). In maize, leaf growth was highly sensitive to alterations of inner plant hydraulic conductance, through pharmacological inhibition of AQPs (Ehlert et al., 2009) or genetic alteration of ABA biosynthesis which in turn altered AQP expression (Parent et al., 2009).

In summary, a hydraulic resistance between vascular and peripheral expanding tissues may result in marked growth-induced water potential gradients, which would in turn collapse cell turgor and result in an immediate growth arrest. Thus, high AQP-mediated cell water permeability can be highly beneficial to enhance cell-to-cell water transport in expanding tissues. Under water stress conditions, however, solute deposition rate in the elongation zone may become the limiting factor to sustain water inflow, turgor and ultimately growth (Fricke and Peters, 2002). There are now numerous reports showing that AQP deregulation can lead to enhancement of plant growth, but the reasons behind must be more complex than a direct alleviation of hydraulic limitations for growth. For instance, overexpression of *At*PIP1;2 in tobacco plants led to a significant increase in plant growth rate, leaf transpiration rate, stomatal density, and photosynthetic efficiency under favorable growing conditions (Aharon et al., 2003). By contrast, these plants showed a very poor response to water deprivation with enhanced leaf wilting, indicating that stomatal deregulation was the primary cause of altered growth in transgenic materials. Some transgenic strategies were more successful to optimize growth in drought conditions. For instance, overexpression of *OsPIP1;3* under the control of a stress-inducible promoter in a drought-sensitive cultivar of rice, resulted in a higher leaf water potential and transpiration rate in water stress conditions (Lian et al., 2004). This indicates that this AQP can indeed play a role in drought resistance and ultimately promote plant growth.

This kind of observations has now found a better interpretation frame by considering the anisohydric vs. isohydric water management strategies (Sade et al., 2012). Isohydric plants exert a strict stomatal control to maintain midday leaf water potential, independent of environmental constraints. Anisohydric plants have a more risky strategy and keep their stomata open under conditions of water shortage, to maintain photosynthetic assimilation and growth, but at the expense of leaf water potential maintenance. This strategy, which requires improved tissue hydraulic performance was associated to enhanced expression of certain tonoplast AQP isoforms in leaves. Overexpression of a TIP homolog in tomato (Sade et al., 2010) increased mesophyll protoplast water permeability and transpiration, especially under water limiting conditions. In addition, a strong relation between *TIP2;1* expression, K_{leaf} and g_s was observed in grapevine under various irrigation regimes (Pou et al., 2013).

AQPs, CARBON FIXATION AND GROWTH

Following the initial phase of turgor-driven cell expansion, a proper supply of carbon and therefore efficient photosynthesis are necessary for new cell wall deposition and an overall increase in dry matter (Pantin et al., 2012). Thus, the ability of some plant AQPs to transport CO_2, in addition to water, may also be highly relevant to their beneficial role in plant growth. In particular, functional expression in oocytes or yeast of a tobacco PIP AQP, *Nt*AQP1, has shown that this AQP can enhance membrane permeability to gaseous CO_2 (Uehlein et al., 2003). Immunological and translational fusion approaches further showed that *Nt*AQP1 was present in guard cells and mesophyll cells, where it localized to both the plasma membrane and in the inner chloroplast membranes. The latter localization is particularly suggestive of a role in CO_2 assimilation (Uehlein and Kaldenhoff, 2008).

In transgenic tobacco plants with altered expression of *NtAQP1*, the rate of ^{14}C incorporation in leaf disks fed with $^{14}CO_2$ (Uehlein et al., 2003), the intensity of gas exchange, chlorophyll fluorescence, and ^{13}C discrimination (Flexas et al., 2006) were positively correlated to the level of *NtAQP1* expression. These results were interpreted to mean that *Nt*AQP1 functions as a CO_2 channel in the mesophyll. These initial observations have now been extended to rice (Hanba et al., 2004) and *Arabidopsis* (Heckwolf et al., 2011). In the latter study, *Arabidopsis pip1;2* knock-out plants displayed a reduction by 40% of their mesophyll conductance (g_m) to CO_2. With respect to previous reports, this work defines a clear molecular and genetic context in which to address the function of PIPs in CO_2 transport. In view of other possible contributors of g_m such as cell walls and carbonic anhydrases (Evans et al., 2009), it remains to be understood, however, how a single AQP isoform can contribute

up to 40% of g_m. Also, it is intriguing that the *At*PIP1;2 isoform was also identified as an important component of root and leaf hydraulics (Postaire et al., 2010). Thus, much remains to be learnt about the interplay and regulation of water and CO_2 transport by AQPs. The possible coupling of tissue hydraulics with growth and carbon assimilation provides unique research perspectives in plant integrative biology.

CONCLUSION

Recent research indicates that the veins, and the AQPs that are expressed in these territories, represent key determinants of leaf hydraulics. Understanding how the vascular architecture of leaves optimizes their hydraulic behavior or, in other words, understanding the adaptive value of leaf venation according to species and/or natural habitats represents an important challenge for future studies. Besides studies on xylem differentiation, a better knowledge of the function and regulation of the numerous AQP homologs expressed in plant leaves is also critically needed to understand how multiple environmental factors such as day/night cycles or water stress act alone or in combination to alter leaf hydraulics. While a role for AQPs in phloem loading, leaf movement and CO_2 transport is emerging, we also anticipate that genetically altered plants will help decipher these and other new AQP functions. Finally, integrative studies have shown how the hydraulics of inner leaf tissues can have a strong impact on the dynamic responses of leaf water potential and stomata, and as a consequence on plant carbon economy and leaf expansion growth. These studies point to the power but also complexity of biotechnological strategies where plant AQP function is manipulated to potentially improve plant growth and tolerance to water stress.

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REFERENCES

Aasamaa, K., Niinemets, U., and Sober, A. (2005). Leaf hydraulic conductance in relation to anatomical and functional traits during *Populus tremula* leaf ontogeny. *Tree Physiol.* 25, 1409–1418. doi: 10.1093/treephys/25.11.1409

Ache, P., Bauer, H., Kollist, H., Al-Rasheid, K. A., Lautner, S., Hartung, W., et al. (2010). Stomatal action directly feeds back on leaf turgor: new insights into the regulation of the plant water status from non-invasive pressure probe measurements. *Plant J.* 62, 1072–1082. doi: 10.1111/j.1365-313X.2010.04213.x

Aharon, R., Shahak, Y., Wininger, S., Bendov, R., Kapulnik, Y., and Galili, G. (2003). Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. *Plant Cell* 15, 439–447. doi: 10.1105/tpc.009225

Alleva, K., Niemietz, C. M., Sutka, M., Maurel, C., Parisi, M., Tyerman, S. D., et al. (2006). Plasma membrane of *Beta vulgaris* storage root shows high water channel activity regulated by cytoplasmic pH and a dual range of calcium concentrations. *J. Exp. Bot.* 57, 609–621. doi: 10.1093/jxb/erj046

Ameglio, T., Ewers, F. W., Cochard, H., Martignac, M., Vandame, M., Bodet, C., et al. (2001). Winter stem xylem pressure in walnut trees: effects of carbohydrates, cooling and freezing. *Tree Physiol.* 21, 387–394. doi: 10.1093/treephys/21.6.387

Amiard, V., Mueh, K. E., Demmig-Adams, B., Ebbert, V., Turgeon, R., and Adams, W. W. III (2005). Anatomical and photosynthetic acclimation to the light environment in species with differing mechanisms of phloem loading. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12968–12973. doi: 10.1073/pnas.0503784102

Baaziz, K. B., Lopez, D., Rabot, A., Combes, D., Gousset, A., Bouzid, S., et al. (2012). Light-mediated K_{leaf} induction and contribution of both the PIP1s and PIP2s aquaporins in five tree species: walnut (*Juglans regia*) case study. *Tree Physiol.* 32, 423–434. doi: 10.1093/treephys/tps022

Barrieu, F., Chaumont, F., and Chrispeels, M. J. (1998). High expression of the tonoplast aquaporin ZmTIP1 in epidermal and conducting tissues of maize. *Plant Physiol.* 117, 1153–1163. doi: 10.1104/pp.117.4.1153

Besse, M., Knipfer, T., Miller, A. J., Verdeil, J. L., Jahn, T. P., and Fricke, W. (2011). Developmental pattern of aquaporin expression in barley (*Hordeum vulgare* L.) leaves. *J. Exp. Bot.* 62, 4127–4142. doi: 10.1093/jxb/err175

Bienert, G. P., Moller, A. L., Kristiansen, K. A., Schulz, A., Moller, I. M., Schjoerring, J. K., et al. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J. Biol. Chem.* 282, 1183–1192. doi: 10.1074/jbc.M603761200

Bienert, G. P., Schussler, M. D., and Jahn, T. P. (2008). Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out. *Trends Biochem. Sci.* 33, 20–26. doi: 10.1016/j.tibs.2007.10.004

Boursiac, Y., Prak, S., Boudet, J., Postaire, O., Luu, D. T., Tournaire-Roux, C., et al. (2008). The response of *Arabidopsis* root water transport to a challenging environment implicates reactive oxygen species- and phosphorylation-dependent internalization of aquaporins. *Plant Signal. Behav.* 3, 1096–1098. doi: 10.4161/psb.3.12.7002

Brodribb, T. J., Bowman, D. J., Nichols, S., Delzon, S., and Burlett, R. (2010). Xylem function and growth rate interact to determine recovery rates after exposure to extreme water deficit. *New Phytol.* 188, 533–542. doi: 10.1111/j.1469-8137.2010.03393.x

Brodribb, T. J., and Feild, T. S. (2010). Leaf hydraulic evolution led a surge in leaf photosynthetic capacity during early angiosperm diversification. *Ecol. Lett.* 13, 175–183. doi: 10.1111/j.1461-0248.2009.01410.x

Brodribb, T. J., Feild, T. S., and Jordan, G. J. (2007). Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiol.* 144, 1890–1898. doi: 10.1104/pp.107.101352

Brodribb, T. J., Holbrook, N. M., Zwieniecki, M. A., and Palma, B. (2005). Leaf hydraulic capacity in ferns, conifers and angiosperms: impacts on photosynthetic maxima. *New Phytol.* 165, 839–846. doi: 10.1111/j.1469-8137.2004.01259.x

Bucci, S. J., Scholz, F. G., Goldstein, G., Meinzer, F. C., Sternberg, L., and Da, S. L. (2003). Dynamic changes in hydraulic conductivity in petioles of two savanna tree species: factors and mechanisms contributing to the refilling of embolized vessels. *Plant Cell Environ.* 26, 1633–1645. doi: 10.1046/j.0140-7791.2003.01082.x

Chaumont, F., Moshelion, M., and Daniels, M. J. (2005). Regulation of plant aquaporin activity. *Biol. Cell* 97, 749–764. doi: 10.1042/BC20040133

Cochard, H., Froux, F., Mayr, S., and Coutand, C. (2004). Xylem wall collapse in water-stressed pine needles. *Plant Physiol.* 134, 401–408. doi: 10.1104/pp.103.028357

Cochard, H., Venisse, J. S., Barigah, T. S., Brunel, N., Herbette, S., Guilliot, A., et al. (2007). Putative role of aquaporins in variable hydraulic conductance of leaves in response to light. *Plant Physiol.* 143, 122–133. doi: 10.1104/pp.106.090092

Cosgrove, D. J. (1987). Wall relaxation and the driving forces for cell expansive growth. *Plant Physiol.* 84, 561–564. doi: 10.1104/pp.84.3.561

Cui, X. H., Hao, F. S., Chen, H., Chen, J., and Wang, X. C. (2008). Expression of the *Vicia faba* VfPIP1 gene in *Arabidopsis thaliana* plants improves their drought resistance. *J. Plant Res.* 121, 207–214. doi: 10.1007/s10265-007-0130-z

Da Ines, O., Graf, W., Franck, K. I., Albert, A., Winkler, J. B., Scherb, H., et al. (2010). Kinetic analyses of plant water relocation using deuterium as tracer - reduced water flux of *Arabidopsis* pip2 aquaporin knockout mutants. *Plant Biol. (Stuttg.)* 12(Suppl. 1), 129–139. doi: 10.1111/j.1438-8677.2010.00385.x

Dynowski, M., Schaaf, G., Loque, D., Moran, O., and Ludewig, U. (2008). Plant plasma membrane water channels conduct the signalling molecule

H_2O_2. *Biochem. J.* 414, 53–61. doi: 10.1042/BJ20080287

Ehlert, C., Maurel, C., Tardieu, F., and Simonneau, T. (2009). Aquaporin-mediated reduction in maize root hydraulic conductivity impacts cell turgor and leaf elongation even without changing transpiration. *Plant Physiol.* 150, 1093–1104. doi: 10.1104/pp.108.131458

Eto, K., Noda, Y., Horikawa, S., Uchida, S., and Sasaki, S. (2010). Phosphorylation of aquaporin-2 regulates its water permeability. *J. Biol. Chem.* 285, 40777–40784. doi: 10.1074/jbc.M110.151928

Evans, J. R., Kaldenhoff, R., Genty, B., and Terashima, I. (2009). Resistances along the CO_2 diffusion pathway inside leaves. *J. Exp. Bot.* 60, 2235–2248. doi: 10.1093/jxb/erp117

Flexas, J., Ribas-Carbo, M., Hanson, D. T., Bota, J., Otto, B., Cifre, J., et al. (2006). Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. *Plant J.* 48, 427–439. doi: 10.1111/j.1365-313X.2006.02879.x

Frangne, N., Maeshima, M., Schaffner, A. R., Mandel, T., Martinoia, E., and Bonnemain, J. L. (2001). Expression and distribution of a vaculoar aquaporin in young and mature leaf tissues of *Brassica napus* in relation to water fluxes. *Planta* 212, 270–278. doi: 10.1007/s004250000390

Franks, P. J. (2003). Use of the pressure probe in studies of stomatal function. *J. Exp. Bot.* 54, 1495–1504. doi: 10.1093/jxb/erg162

Fraysse, L. C., Wells, B., Mccann, M. C., and Kjellbom, P. (2005). Specific plasma membrane aquaporins of the PIP1 subfamily are expressed in sieve elements and guard cells. *Biol. Cell.* 97, 519–534. doi: 10.1042/BC20040122

Fricke, W. (2002). Biophysical limitation of cell elongation in cereal leaves. *Ann. Bot.* 90, 157–167. doi: 10.1093/aob/mcf180

Fricke, W., and Peters, W. S. (2002). The biophysics of leaf growth in salt-stressed barley. A study at the cell level. *Plant Physiol.* 129, 374–388. doi: 10.1104/pp.001164

Galmés, J., Pou, A., Alsina, M. M., Tomas, M., Medrano, H., and Flexas, J. (2007). Aquaporin expression in response to different water stress intensities and recovery in Richter-110 (*Vitis sp.*): relationship with ecophysiological status. *Planta* 226, 671–681.

Gerbeau, P., Amodeo, G., Henzler, T., Santoni, V., Ripoche, P., and Maurel, C. (2002). The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. *Plant J.* 30, 71–81. doi: 10.1046/j.1365-313X.2002.01268.x

Gilliham, M., Dayod, M., Hocking, B. J., Xu, B., Conn, S. J., Kaiser, B. N., et al. (2011). Calcium delivery and storage in plant leaves: exploring the link with water flow. *J. Exp. Bot.* 62, 2233–2250. doi: 10.1093/jxb/err111

Guyot, G., Scoffoni, C., and Sack, L. (2012). Combined impacts of irradiance and dehydration on leaf hydraulic conductance: insights into vulnerability and stomatal control. *Plant Cell Environ.* 35, 857–871. doi: 10.1111/j.1365-3040.2011.02458.x

Hachez, C., Heinen, R. B., Draye, X., and Chaumont, F. (2008). The expression pattern of plasma membrane aquaporins in maize leaf highlights their role in hydraulic regulation. *Plant Mol. Biol.* 68, 337–353. doi: 10.1007/s11103-008-9373-x

Hachez, C., Moshelion, M., Zelazny, E., Cavez, D., and Chaumont, F. (2006). Localization and quantification of plasma membrane aquaporin expression in maize primary root: a clue to understanding their role as cellular plumbers. *Plant Mol. Biol.* 62, 305–323. doi: 10.1007/s11103-006-9022-1

Hanba, Y. T., Shibasaka, M., Hayashi, Y., Hayakawa, T., Kasamo, K., Terashima, I., et al. (2004). Overexpression of the barley aquaporin HvPIP2;1 increases internal CO2 conductance and CO2 assimilation in the leaves of transgenic rice plants. *Plant Cell Physiol.* 45, 521–529. doi: 10.1093/pcp/pch070

Heckwolf, M., Pater, D., Hanson, D. T., and Kaldenhoff, R. (2011). The *Arabidopsis thaliana* aquaporin AtPIP1;2 is a physiologically relevant CO_2 transport facilitator. *Plant J.* 67, 795–804. doi: 10.1111/j.1365-313X.2011.04634.x

Hedfalk, K., Törnroth-Horsefield, S., Nyblom, M., Johanson, U., Kjellbom, P., and Neutze, R. (2006). Aquaporin gating. *Curr. Opin. Struct. Biol.* 16, 447–456.

Heinen, R. B., Ye, Q., and Chaumont, F. (2009). Role of aquaporins in leaf physiology. *J. Exp. Bot.* 60, 2971–2985. doi: 10.1093/jxb/erp171

Henzler, T., Ye, Q., and Steudle, E. (2004). Oxidative gating of water channels (aquaporins) in Chara by hydroxyl radicals. *Plant Cell Environ.* 27, 1184–1195. doi: 10.1111/j.1365-3040.2004.01226.x

Jang, J. Y., Kim, D. G., Kim, Y. O., Kim, J. S., and Kang, H. (2004). An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. *Plant Mol. Biol.* 54, 713–725. doi: 10.1023/B:PLAN.0000040900.61345.a6

Johansson, I., Larsson, C., Ek, B., and Kjellbom, P. (1996). The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. *Plant Cell* 8, 1181–1191.

Johnson, D. M., Meinzer, F. C., Woodruff, D. R., and Mcculloh, K. A. (2009). Leaf xylem embolism, detected acoustically and by cryo-SEM, corresponds to decreases in leaf hydraulic conductance in four evergreen species. *Plant Cell Environ.* 32, 828–836. doi: 10.1111/j.1365-3040.2009.01961.x

Kaldenhoff, R., Grote, K., Zhu, J. J., and Zimmermann, U. (1998). Significance of plasmalemma aquaporins for water-transport in *Arabidopsis thaliana*. *Plant J.* 14, 121–128. doi: 10.1046/j.1365-313X.1998.00111.x

Kaldenhoff, R., Kolling, A., Meyers, J., Karmann, U., Ruppel, G., and Richter, G. (1995). The blue light-responsive AthH2 gene of *Arabidopsis thaliana* is primarily expressed in expanding as well as in differentiating cells and encodes a putative channel protein of the plasmalemma. *Plant J.* 7, 87–95. doi: 10.1046/j.1365-313X.1995.07010087.x

Kaldenhoff, R., Ribas-Carbo, M., Sans, J. F., Lovisolo, C., Heckwolf, M., and Uehlein, N. (2008). Aquaporins and plant water balance. *Plant Cell Environ.* 31, 658–666. doi: 10.1111/j.1365-3040.2008.01792.x

Kim, Y. X., and Steudle, E. (2007). Light and turgor affect the water permeability (aquaporins) of parenchyma cells in the midrib of leaves of *Zea mays*. *J. Exp. Bot.* 58, 4119–4129. doi: 10.1093/jxb/erm270

Kim, Y. X., and Steudle, E. (2009). Gating of aquaporins by light and reactive oxygen species in leaf parenchyma cells of the midrib of *Zea mays*. *J. Exp. Bot.* 60, 547–556. doi: 10.1093/jxb/ern299

Kirch, H. H., Vera-Estrella, R., Golldack, D., Quigley, F., Michalowski, C. B., Barkla, B. J., et al. (2000). Expression of water channel proteins in *Mesembryanthemum crystallinum*. *Plant Physiol.* 123, 111–124. doi: 10.1104/pp.123.1.111

Kline, K. G., Barrett-Wilt, G. A., and Sussman, M. R. (2010). In planta changes in protein phosphorylation induced by the plant hormone abscisic acid. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15986-15991. doi: 10.1073/pnas.1007879107

Kuwagata, T., Ishikawa-Sakurai, J., Hayashi, H., Nagasuga, K., Fukushi, K., Ahamed, A., et al. (2012). Influence of low air humidity and low root temperature on water uptake, growth and aquaporin expression in rice plants. *Plant Cell Physiol.* 53, 1418–1431. doi: 10.1093/pcp/pcs087

Lee, S. H., Chung, C. G., and Zwiazek, J. J. (2009). Effects of irradiance on cell water relations in leaf bundle sheath cells of wild-type and transgenic tobacco (*Nicotiana tabacum*) plants overexpressing aquaporins. *Plant Sci.* 176, 248–255. doi: 10.1016/j.plantsci.2008.10.013

Leegood, R. C. (2008). Roles of the bundle sheath cells in leaves of C3 plants. *J. Exp. Bot.* 59, 1663–1673. doi: 10.1093/jxb/erm335

Lersten, N., and Curtis, J. (1997). Anatomy and distribution of foliar idioblasts in *Scrophularia* and *Verbascum* (*Scrophulariaceae*). *Am. J. Bot.* 84, 1638. doi: 10.2307/2446461

Levin, M., Lemcoff, J. H., Cohen, S., and Kapulnik, Y. (2007). Low air humidity increases leaf-specific hydraulic conductance of *Arabidopsis thaliana* (L.) Heynh (Brassicaceae). *J. Exp. Bot.* 58, 3711–3718. doi: 10.1093/jxb/erm220

Lewis, A. M., and Boose, E. R. (1995). Estimating volume flow rates through xylem conduits. *Am. J. Bot.* 82, 1112–1116. doi: 10.2307/2446063

Lian, H. L., Yu, X., Ye, Q., Ding, X., Kitagawa, Y., Kwak, S. S., et al. (2004). The role of aquaporin RWC3 in drought avoidance in rice. *Plant Cell Physiol.* 45, 481–489. doi: 10.1093/pcp/pch058

Lo Gullo, M. A., Nardini, A., Trifilo, P., and Salleo, S. (2005). Diurnal and seasonal variations in leaf hydraulic conductance in evergreen and deciduous trees. *Tree Physiol.* 25, 505–512. doi: 10.1093/treephys/25.4.505

Ludevid, D., Hofte, H., Himelblau, E., and Chrispeels, M. J. (1992). The expression pattern of the Tonoplast Intrinsic Protein gamma-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. *Plant Physiol.* 100, 1633–1639. doi: 10.1104/pp.100.4.1633

Martre, P., Morillon, R., Barrieu, F., North, G. B., Nobel, P. S., and Chrispeels, M. J. (2002). Plasma membrane aquaporins play a significant role during recovery from water deficit. *Plant Physiol.* 130, 2101–2110. doi: 10.1104/pp.009019

Maurel, C., Verdoucq, L., Luu, D. T., and Santoni, V. (2008). Plant aquaporins: membrane channels with multiple integrated functions. *Annu. Rev. Plant Biol.* 59, 595–624. doi:

10.1146/annurev.arplant.59.032607.092734

McKown, A. D., Cochard, H., and Sack, L. (2010). Decoding leaf hydraulics with a spatially explicit model: principles of venation architecture and implications for its evolution. *Am. Nat.* 175, 447–460. doi: 10.1086/650721

Mirzaei, M., Pascovici, D., Atwell, B. J., and Haynes, P. A. (2012). Differential regulation of aquaporins, small GTPases and V-ATPases proteins in rice leaves subjected to drought stress and recovery. *Proteomics* 12, 864–877. doi: 10.1002/pmic.201100389

Monneuse, J. M., Sugano, M., Becue, T., Santoni, V., Hem, S., and Rossignol, M. (2011). Towards the profiling of the *Arabidopsis thaliana* plasma membrane transportome by targeted proteomics. *Proteomics* 11, 1789–1797. doi: 10.1002/pmic.201000660

Montalvo-Hernandez, L., Piedra-Ibarra, E., Gomez-Silva, L., Lira-Carmona, R., Acosta-Gallegos, J. A., Vazquez-Medrano, J., et al. (2008). Differential accumulation of mRNAs in drought-tolerant and susceptible common bean cultivars in response to water deficit. *New Phytol.* 177, 102–113.

Morillon, R., and Chrispeels, M. J. (2001). The role of ABA and the transpiration stream in the regulation of the osmotic water permeability of leaf cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14138–14143. doi: 10.1073/pnas.231471998 PMCid:150592

Moshelion, M., Becker, D., Biela, A., Uehlein, N., Hedrich, R., Otto, B., et al. (2002). Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. *Plant Cell* 14, 727–739. doi: 10.1105/tpc.010351

Moshelion, M., Moran, N., and Chaumont, F. (2004). Dynamic changes in the osmotic water permeability of protoplast plasma membrane. *Plant Physiol.* 135, 2301–2317. doi: 10.1104/pp.104.043000

Nardini, A., Raimondo, F., Lo Gullo, M. A., and Salleo, S. (2010). Leafminers help us understand leaf hydraulic design. *Plant Cell Environ.* 33, 1091–1100. doi: 10.1111/j.1365-3040.2010.02131.x

Nardini, A., and Salleo, S. (2005). Water stress-induced modifications of leaf hydraulic architecture in sunflower: co-ordination with gas exchange. *J. Exp. Bot.* 56, 3093–3101. doi: 10.1093/jxb/eri306

Nardini, A., Salleo, S., and Raimondo, F. (2003). Changes in leaf hydraulic conductance correlate with leaf vein embolism in *Cercis siliquastrum* L. *Trees* 17, 529–534. doi: 10.1007/s00468-003-0265-z

Oliviusson, P., Salaj, J., and Hakman, I. (2001). Expression pattern of transcripts encoding water channel-like proteins in Norway spruce (*Picea abies*). *Plant Mol. Biol.* 46, 289–299. doi: 10.1023/A:1010611605142

Otto, B., and Kaldenhoff, R. (2000). Cell-specific expression of the mercury-insensitive plasma-membrane aquaporin NtAQP1 from *Nicotiana tabacum*. *Planta* 211, 167–172. doi: 10.1007/s004250000275

Pantin, F., Monnet, F., Jannaud, D., Costa, J. M., Renaud, J., Muller, B., et al. (2013). The dual effect of abscisic acid on stomata. *New Phytol.* 197, 65–72. doi: 10.1111/nph.12013

Pantin, F., Simonneau, T., and Muller, B. (2012). Coming of leaf age: control of growth by hydraulics and metabolics during leaf ontogeny. *New Phytol.* 196, 349–366. doi: 10.1111/j.1469-8137.2012.04273.x

Parent, B., Hachez, C., Redondo, E., Simonneau, T., Chaumont, F., and Tardieu, F. (2009). Drought and abscisic acid effects on aquaporin content translate into changes in hydraulic conductivity and leaf growth rate: a trans-scale approach. *Plant Physiol.* 149, 2000–2012. doi: 10.1104/pp.108.130682

Phillips, A. L., and Huttly, A. K. (1994). Cloning of two gibberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: expression of the tonoplast water channel, gamma-TIP, is increased by GA3. *Plant Mol. Biol.* 24, 603–615. doi: 10.1007/BF00023557

Postaire, O., Tournaire-Roux, C., Grondin, A., Boursiac, Y., Morillon, R., Schaffner, A. R., et al. (2010). A PIP1 aquaporin contributes to hydrostatic pressure-induced water transport in both the root and rosette of *Arabidopsis*. *Plant Physiol.* 152, 1418–1430. doi: 10.1104/pp.109.145326

Pou, A., Medrano, H., Flexas, J., and Tyerman, S. D. (2013). A putative role for TIP and PIP aquaporins in dynamics of leaf hydraulic and stomatal conductances in grapevine under water stress and re-watering. *Plant Cell Environ.* 36, 828–843. doi: 10.1111/pce.12019

Prado, K., Boursiac, Y., Tournaire-Roux, C., Monneuse, J. M., Postaire, O., Da Ines, O., et al. (2013). Regulation of *Arabidopsis* leaf hydraulics involves light-dependent phosphorylation of aquaporins in veins. *Plant Cell* 25, 1029–1039. doi: 10.1105/tpc.112.108456

Prak, S., Hem, S., Boudet, J., Viennois, G., Sommerer, N., Rossignol, M., et al. (2008). Multiple phosphorylations in the C-terminal tail of plant plasma membrane aquaporins: role in subcellular trafficking of AtPIP2;1 in response to salt stress. *Mol. Cell. Proteomics* 7, 1019–1030. doi: 10.1074/mcp.M700566-MCP200

Ramahaleo, T., Morillon, R., Alexandre, J., and Lassalles, J.-P. (1999). Osmotic water permeability of isolated protoplasts. Modifications during development. *Plant Physiol.* 119, 885–896. doi: 10.1104/pp.119.3.885

Rockwell, F. E., Holbrook, N. M., and Zwieniecki, M. A. (2011). Hydraulic conductivity of red oak (*Quercus rubra* L.) leaf tissue does not respond to light. *Plant Cell Environ.* 34, 565–579. doi: 10.1111/j.1365-3040.2011.02263.x

Roth-Nebelsick, A., Uhl, D., Mosbrugger, V., and Kerp, H. (2001). Evolution and function of leaf venation architecture: a review. *Ann. Bot.* 87, 553–566. doi: 10.1006/anbo.2001.1391

Sack, L., Cowan, P. D., Jaikumar, N., and Holbrook, N. M. (2003). The "hydrology" of leaves: co-ordination of structure and function in temperate woody species. *Plant Cell Environ.* 26, 1343–1356. doi: 10.1046/j.0016-8025.2003.01058.x

Sack, L., and Frole, K. (2006). Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* 87, 483–491. doi: 10.1890/05-0710

Sack, L., and Holbrook, N. M. (2006). Leaf hydraulics. *Annu. Rev. Plant Biol.* 57, 361–381. doi: 10.1146/annurev.arplant.56.032604.144141

Sack, L., Melcher, P. J., Zwieniecki, M. A., and Holbrook, N. M. (2002). The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. *J. Exp. Bot.* 53, 2177–2184. doi: 10.1093/jxb/erf069

Sack, L., and Scoffoni, C. (2012). Measurement of leaf hydraulic conductance and stomatal conductance and their responses to irradiance and dehydration using the Evaporative Flux Method (EFM). *J. Vis. Exp.* 70, 4179. doi: 10.3791/4179

Sack, L., and Scoffoni, C. (2013). Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytol.* 198, 983-1000 doi: 10.1111/nph.12253

Sack, L., Scoffoni, C., Mckown, A. D., Frole, K., Rawls, M., Havran, J. C., et al. (2012). Developmentally based scaling of leaf venation architecture explains global ecological patterns. *Nat. Commun.* 3, 837. doi: 10.1038/ncomms1835

Sack, L., Streeter, C. M., and Holbrook, N. M. (2004). Hydraulic analysis of water flow through leaves of sugar maple and red oak. *Plant Physiol.* 134, 1824–1833. doi: 10.1104/pp.103.031203

Sack, L., Tyree, M. T., and Holbrook, N. M. (2005). Leaf hydraulic architecture correlates with regeneration irradiance in tropical rainforest trees. *New Phytol.* 167, 403–413. doi: 10.1111/j.1469-8137.2005.01432.x

Sade, N., Gebremedhin, A., and Moshelion, M. (2012). Risk-taking plants: anisohydric behavior as a stress-resistance trait. *Plant Signal. Behav.* 7, 767–770. doi: 10.4161/psb.20505

Sade, N., Gebretsadik, M., Seligmann, R., Schwartz, A., Wallach, R., and Moshelion, M. (2010). The role of tobacco Aquaporin1 in improving water use efficiency, hydraulic conductivity, and yield production under salt stress. *Plant Physiol.* 152, 245–254. doi: 10.1104/pp.109.145854

Sakr, S., Alves, G., Morillon, R., Maurel, K., Decourteix, M., Guilliot, A., et al. (2003). Plasma membrane aquaporins are involved in winter embolism recovery in walnut tree. *Plant Physiol.* 133, 630–641. doi: 10.1104/pp.103.027797

Sakurai, J., Ahamed, A., Murai, M., Maeshima, M., and Uemura, M. (2008). Tissue and cell-specific localization of rice aquaporins and their water transport activities. *Plant Cell Physiol.* 49, 30–39. doi: 10.1093/pcp/pcm162

Savvides, A., Fanourakis, D., and Van Ieperen, W. (2012). Co-ordination of hydraulic and stomatal conductances across light qualities in cucumber leaves. *J. Exp. Bot.* 63, 1135–1143. doi: 10.1093/jxb/err348

Secchi, F., and Zwieniecki, M. A. (2010). Patterns of PIP gene expression in *Populus trichocarpa* during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily as moderators of refilling process. *Plant Cell Environ.* 33, 1285–1297. doi: 10.1111/j.1365-3040.2010.02147.x

Sellin, A., Ounapuu, E., and Karusion, A. (2010). Experimental evidence supporting the concept of light-mediated modulation of stem hydraulic conductance. *Tree Physiol.* 30, 1528–1535. doi: 10.1093/treephys/tpq091

Sellin, A., Sack, L., Ounapuu, E., and Karusion, A. (2011). Impact of light quality on leaf and shoot hydraulic

properties: a case study in silver birch (*Betula pendula*). *Plant Cell Environ.* 34, 1079–1087. doi: 10.1111/j.1365-3040.2011.02306.x

Shatil-Cohen, A., Attia, Z., and Moshelion, M. (2011). Bundle-sheath cell regulation of xylem-mesophyll water transport via aquaporins under drought stress: a target of xylem-borne ABA? *Plant J.* 67, 72–80. doi: 10.1111/j.1365-313X.2011.04576.x

Siefritz, F., Otto, B., Bienert, G. P., Van Der Krol, A., and Kaldenhoff, R. (2004). The plasma membrane aquaporin NtAQP1 is a key component of the leaf unfolding mechanism in tobacco. *Plant J.* 37, 147–155. doi: 10.1046/j.1365-313X.2003.01947.x

Sjövall-Larsen, S., Alexandersson, E., Johansson, I., Karlsson, M., Johanson, U., and Kjellbom, P. (2006). Purification and characterization of two protein kinases acting on the aquaporin SoPIP2;1. *Biochim. Biophys. Acta* 1758, 1157–1164.

Steudle, E. (1989). Water flow in plants and its coupling to other processes: an overview. *Methods Enzymol.* 174, 183–225. doi: 10.1016/0076-6879(89)74019-2

Steudle, E., and Peterson, C. A. (1998). How does water get through roots? *J. Exp. Bot.* 49, 775–788.

Tang, A. C., and Boyer, J. S. (2002). Growth-induced water potentials and the growth of maize leaves. *J. Exp. Bot.* 53, 489–503. doi: 10.1093/jexbot/53.368.489

Törnroth-Horsefield, S., Wang, Y., Hedfalk, K., Johanson, U., Karlsson, M., Tajkhorshid, E., et al. (2006). Structural mechanism of plant aquaporin gating. *Nature* 439, 688–694.

Tournaire-Roux, C., Sutka, M., Javot, H., Gout, E., Gerbeau, P., Luu, D.-T., et al. (2003). Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. *Nature* 425, 393–397. doi: 10.1038/nature01853

Tsuda, M., and Tyree, M. T. (2000). Plant hydraulic conductance measured by the high pressure flow meter in crop plants. *J. Exp. Bot.* 51, 823–828. doi: 10.1093/jexbot/51.345.823

Tyerman, S. D., Niemietz, C. M., and Bramley, H. (2002). Plant aquaporins: multifunctional water and solute channels with expanding roles. *Plant Cell Environ.* 25, 173–194. doi: 10.1046/j.0016-8025.2001.00791.x

Tyree, M. T., Nardini, A., Salleo, S., Sack, L., and El Omari, B. (2005). The dependence of leaf hydraulic conductance on irradiance during HPFM measurements: any role for stomatal response? *J. Exp. Bot.* 56, 737–744. doi: 10.1093/jxb/eri045

Uehlein, N., and Kaldenhoff, R. (2008). Aquaporins and plant leaf movements. *Ann. Bot.* 101, 1–4. doi: 10.1093/aob/mcm278

Uehlein, N., Lovisolo, C., Siefritz, F., and Kaldenhoff, R. (2003). The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature* 425, 734–737. doi: 10.1038/nature02027

Verdoucq, L., Grondin, A., and Maurel, C. (2008). Structure–function analysis of plant aquaporin AtPIP2;1 gating by divalent cations and protons. *Biochem. J.* 415, 409–416. doi: 10.1042/BJ20080275

Voicu, M. C., Cooke, J. E., and Zwiazek, J. J. (2009). Aquaporin gene expression and apoplastic water flow in bur oak (*Quercus macrocarpa*) leaves in relation to the light response of leaf hydraulic conductance. *J. Exp. Bot.* 60, 4063–4075. doi: 10.1093/jxb/erp239

Voicu, M. C., and Zwiazek, J. J. (2010). Inhibitor studies of leaf lamina hydraulic conductance in trembling aspen (*Populus tremuloides Michx.*) leaves. *Tree Physiol.* 30, 193–204. doi: 10.1093/treephys/tpp112

Voicu, M. C., Zwiazek, J. J., and Tyree, M. T. (2008). Light response of hydraulic conductance in bur oak (*Quercus macrocarpa*) leaves. *Tree Physiol.* 28, 1007–1015. doi: 10.1093/treephys/28.7.1007

Volkov, V., Hachez, C., Moshelion, M., Draye, X., Chaumont, F., and Fricke, W. (2007). Water permeability differs between growing and non-growing barley leaf tissues. *J. Exp. Bot.* 58, 377–390. doi: 10.1093/jxb/erl203

Wang, Y., and Tajkhorshid, E. (2007). Molecular mechanisms of conduction and selectivity in aquaporin water channels. *J. Nutr.* 137, 1509S–1515S; discussion 1516S–1517S.

Wei, C., Tyree, M. T., and Steudle, E. (1999). Direct measurement of xylem pressure in leaves of intact maize plants. A test of the cohesion-tension theory taking hydraulic architecture into consideration. *Plant Physiol.* 121, 1191–1206. doi: 10.1104/pp.121.4.1191

Wei, W., Alexandersson, E., Golldack, D., Miller, A. J., Kjellbom, P. O., and Fricke, W. (2007). HvPIP1;6, a barley (*Hordeum vulgare* L.) plasma membrane water channel particularly expressed in growing compared with non-growing leaf tissues. *Plant Cell Physiol.* 48, 1132–1147. doi: 10.1093/pcp/pcm083

Ye, Q., Holbrook, N. M., and Zwieniecki, M. A. (2008). Cell-to-cell pathway dominates xylem-epidermis hydraulic connection in *Tradescantia fluminensis* (Vell. Conc.) leaves. *Planta* 227, 1311–1319. doi: 10.1007/s00425-008-0703-7

Yue, X., Zhao, X., Fei, Y., and Zhang, X. (2012). Correlation of aquaporins and transmembrane solute transporters revealed by genome-wide analysis in developing maize leaf. *Comp. Funct. Genomics* 2012, 546930. doi: 10.1155/2012/546930

Zwieniecki, M. A., Brodribb, T. J., and Holbrook, N. M. (2007). Hydraulic design of leaves: insights from rehydration kinetics. *Plant Cell Environ.* 30, 910–921. doi: 10.1111/j.1365-3040.2007.001681.x

Zwieniecki, M. A., Melcher, P. J., Boyce, C. K., Sack, L., and Holbrook, N. M. (2002). Hydraulic architecture of leaf venation in *Laurus nobilis* L. *Plant Cell Environ.* 25, 1445–1450. doi: 10.1046/j.1365-3040.2002.00922.x

Zwieniecki, M. A., Melcher, P. J., and Holbrook, N. M. (2001). Hydrogel control of xylem hydraulic resistance in plants. *Science* 291, 1059–1062. doi: 10.1126/science.1057175

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Multifunctional fructans and raffinose family oligosaccharides

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Fructans and raffinose family oligosaccharides (RFOs) are the two most important classes of water-soluble carbohydrates in plants. Recent progress is summarized on their metabolism (and regulation) and on their functions in plants and in food (prebiotics, antioxidants). Interest has shifted from the classic inulin-type fructans to more complex fructans. Similarly, alternative RFOs were discovered next to the classic RFOs. Considerable progress has been made in the understanding of structure–function relationships among different kinds of plant fructan metabolizing enzymes. This helps to understand their evolution from (invertase) ancestors, and the evolution and role of so-called "defective invertases." Both fructans and RFOs can act as reserve carbohydrates, membrane stabilizers and stress tolerance mediators. Fructan metabolism can also play a role in osmoregulation (e.g., flower opening) and source–sink relationships. Here, two novel emerging roles are highlighted. First, fructans and RFOs may contribute to overall cellular reactive oxygen species (ROS) homeostasis by specific ROS scavenging processes in the vicinity of organellar membranes (e.g., vacuole, chloroplasts). Second, it is hypothesized that small fructans and RFOs act as phloem-mobile signaling compounds under stress. It is speculated that such underlying antioxidant and oligosaccharide signaling mechanisms contribute to disease prevention in plants as well as in animals and in humans.

Keywords: antioxidant, fructan, immunity, oligosaccharide, raffinose, signaling, stress, sucrose

INTRODUCTION

Sucrose (Suc; Glcα1,2βFru) takes a central position in plant metabolism as the first free sugar formed during photosynthesis and the major transport compound to bring carbon skeletons from source to sink tissues (Koch, 2004). Suc is the substrate for the synthesis of different types of Suc-derived oligosaccharides (Keller and Pharr, 1996). Among those, fructans and raffinose family oligosaccharides (RFOs) are the most important two classes of water-soluble carbohydrates in the plant kingdom. Fructans are fructose (Fru)-based oligo- and polysaccharides, representing the major reserve carbohydrates in about 15% of flowering plant species (Hendry, 1993). Fructans can be linear or branched and their degree of polymerization (DP) ranges from three up to a few hundred, depending on the species, developmental stage and environmental conditions (Van den Ende et al., 2002a). Fructans are classified according to differences in glycosidic linkages [β(2,1), β(2,6) or both]. The best studied fructans are the linear inulin-type fructans (occurring in Asterales such as Jerusalem artichoke and chicory) consisting of β(2,1)-linked Fru units attached to the Suc starter unit. The trisaccharide 1-kestotriose (older nomenclature 1-kestose:Glcα1,2βFru1,2βFru) is the essential building block in this case. However, Fru-only versions of these fructans, lacking a terminal glucose (Glc), and termed inulo-*n*-oses, can also be found under some conditions (Van den Ende et al., 1996). The smallest representatives of these series are inulobiose (Fru1,2βFru) and inulotriose (Fru1,2βFru1,2βFru). The building block for the linear levan-type fructans (also termed phleins in plants) is 6-kestotriose (older nomenclature 6-kestose: Glcα1,2βFru6,2βFru) which is further elongated to polymers with β(2,6)-linkages. They typically occur in forage grasses such as *Dactylis glomerata*, *Phleum pratense*, and *Poa secunda* (Chatterton et al., 1993; Chatterton and Harrison, 1997; Tamura et al., 2009). Graminan-type fructans contain both β(2,1) and β(2,6) linkages. They occur in cereals such as wheat and barley (Van den Ende et al., 2003). Even more complex fructans, based on the 6G-kestotriose backbone (older nomenclature neokestose: Fru2,6Glcα1,2βFru), with further Fru elongations on both sides, occur for instance in oat, *Asparagus*, *Agave*, and in *Lolium* sp. (Pavis et al., 2001). These are termed neo-inulin [predominant β(2,1) linkages] and neo-levan-type [predominant β(2,6) linkages] fructans, respectively. The longstanding view that graminan- and levan-type fructans only occur in monocots has been overruled, since both types have been recently found in *Pachysandra terminalis*, an evergreen, frost-hardy basal eudicot species (Van den Ende et al., 2011a). Although some suggestions were made to link fructan structure to functionality in stress tolerance responses (Valluru and Van den Ende, 2008; Lasseur et al., 2011; Van den Ende et al., 2011a), these relationships are

Abbreviations: 1-FFT, fructan:fructan 1-fructosyl transferase; 1-SST, sucrose:sucrose 1-fructosyl transferase; 6G-FFT, fructan:fructan 6G-fructosyl transferase; 6-SFT, sucrose:fructan 6-fructosyl transferase; CWIs, cell wall invertases; DP, degree of polymerization; FEH, fructan exohydrolase; Fru, fructose; FTs, fructosyltransferases; Gal, galactose; GGTs, galactan:galactan galactosyl transferases; GHXX, family XX of glycoside hydrolases; Glc, glucose; Gol, galactinol; GolS, galactinol synthase; pFT, preliminary fructosyl transferase; pI, iso-electric point; Raf, raffinose; RafS, raffinose synthase; RFOs, Raffinose Family Oligosaccharides; ROS, reactive oxygen species; Sta, stachyose; StaS, stachyose synthase; Suc, sucrose; VIs, vacuolar invertases.

unclear and require further experimental verification. Fructans are believed to accumulate in vacuoles (Wiemken et al., 1986) but it was proposed that, under stress, tonoplast-derived vesicles may transport fructans from the vacuole to the apoplast (Livingston and Henson, 1998; Valluru et al., 2008).

The "classic" RFOs are soluble, non-reducing α(1,6) galactosyl (Gal) extensions of Suc. The trisaccharide raffinose (Raf; Galα1,6Glcα1,2βFru) is the smallest RFO and ubiquitous in the plant kingdom (Keller and Pharr, 1996). Further elongation with Gal residues leads to the DP4 stachyose (Sta; Galα1,6Galα1,6Glcα1,2βFru), verbascose (DP5), ajugose (DP6), etc. Classic RFOs with a DP up to 15 have been found after cold treatment in *Ajuga reptans* L. (Bachmann et al., 1994), a typical RFO accumulator belonging to the *Lamiaceae*. While Raf and Sta occur in all plant parts in genuine RFO accumulators, the higher homologous are usually restricted to the storage organs. Often Sta is the quantitatively dominating carbohydrate in such storage organs (Kandler and Hopf, 1984). Raf and Sta are also important transport compounds in the orders *Lamiales*, *Cucurbitales*, *Cornales*, and in one family of the *Celastrales* (Zimmermann and Ziegler, 1975; Haritatos et al., 1996; Hoffmann-Thoma et al., 1996; Turgeon et al., 2001). Recently, research has been devoted to so-called "alternative" RFOs in plants. These novel plant Gal oligosaccharides did not derive much attention in the past. Among these, the Sta derivative manninotriose (Galα1,6Galα1,6Glc) was found to be the predominant carbohydrate in cold-induced early spring red deadnettle (dos Santos et al., 2013), a unique feature since this compound was never observed before in any RFO accumulator (dos Santos et al., 2013). Intriguingly, Sta does not occur within the *Caryophyllaceae*. Instead, Raf is elongated to the DP4 lychnose (Galα1,6Glcα1,2βFru1,1Gal) and the DP5 stellariose ([6Galα1,6&Galα1,4]Glcα1,2βFru1,1Gal) in cold-treated *Stellaria media* (Vanhaecke et al., 2006, 2008, 2010).

METABOLISM AND ITS REGULATION

Sucrose is not only needed as a substrate for fructan biosynthetic enzymes (termed fructosyltransferases: FTs), organ-specific Suc thresholds trigger the expression of genes encoding FTs (Lu et al., 2002; Maleux and Van den Ende, 2007) and RFO biosynthesis genes (Nägele and Heyer, 2013). Similar to the induction of anthocyanins in *Arabidopsis* (a non-fructan accumulator), it is well-known that fructan synthesis is controlled by a Suc-specific pathway (Bolouri Moghaddam and Van den Ende, 2013a and references therein), which means that the same effects cannot be obtained by using a mixture of Glc and Fru. Calcium, protein kinases and phosphatases are also involved in this inductive process (Martínez-Noël et al., 2009, 2010). Recently, the transcription factor TaMYB13 was found to be an important player in the process leading to FT induction and fructan synthesis in wheat (Xue et al., 2011) but further research into this pathway is needed to fully understand where this transcription factor is situated in the pathway. Even less is known about the pathway leading to RFO synthesis. However, it seems that heat shock transcription factors (HSFs), C-repeat binding factor/drought response element binding factor 1 (CBF/DREB1) type transcription factors and WRKY type of transcription factors (Panikulangara et al., 2004; Ogawa et al., 2007; Wang et al., 2009). Recently, it was reported that target of rapamycin kinase complexes stimulate the pathway leading to RFO synthesis in *Arabidopsis* (Dobrenel et al., 2013 and references therein).

Inulin-type fructans are biosynthesized from Suc by two FTs. First, 1-kestotriose is produced by the activity of a sucrose:sucrose 1-fructosyl transferase (1-SST) which transfers a fructosyl residue from a donor to an acceptor Suc. Then, a fructan:fructan 1-fructosyl transferase (1-FFT) polymerizes 1-kestotriose into higher DP inulin-type fructans (Edelman and Jefford, 1968; Van Laere and Van den Ende, 2002). Sucrose:fructan 6-fructosyl transferases (6-SFTs) are able to introduce branching. They preferentially transfer a fructosyl group from Suc as a donor substrate to 1-kestotriose as acceptor substrate, producing 1&6-kestotetraose (also termed bifurcose), the smallest graminan-type of fructan with mixed-type of linkages. Bifurcose can be further elongated by 6-SFT and 1-FFT, leading to branched, higher DP graminan-type of fructans (Yoshida et al., 2007). However, some of these 6-SFT enzymes might use Suc and/or 6-kestotriose as preferential acceptors, producing levan-type fructans (Tamura et al., 2009). Such 6-SST/6-SFT is also involved in fructan synthesis in *Pachysandra terminalis*, although this particular enzyme also shows extensive hydrolytic activities as well (Van den Ende et al., 2011a; Lammens et al., 2012), and it can be considered as a "premature" FT [preliminary fructosyl transferase (pFT); see also below]. Finally, the enzyme fructan:fructan 6G-fructosyl transferase (6G-FFT) synthesizes 6G-kestotriose (neokestose) from 1-kestotriose as donor substrate and Suc as acceptor substrate. Further elongation by 1-FFT and 6-SFT leads to the formation of inulin- and levan neoseries, respectively (Vijn and Smeekens, 1999). Plants use an array of different fructan exohydrolases (FEHs) to degrade their fructans (Van den Ende et al., 2004; Yoshida et al., 2007; Zhang et al., 2008), including 1-FEHs [preferentially attacking β(2,1) Fru linkages], 6-FEHs [preferentially attacking β (2,6) Fru linkages] and 6&1-FEHs (attacking both types of linkages). These enzymes remove, one by one, terminal Fru units from fructan chains. In contrast to invertases, FEHs cannot use Suc as a substrate. Instead, many FEHs are directly inhibited by Suc at the enzyme level (Verhaest et al., 2007), which represents one of the most important ways of regulation, next to the control of FEH gene expression at the transcriptional level (Van den Ende et al., 2002a). Remarkably, some of the apoplastic localized FEHs show an extreme specificity for single fructan kestotrioses, and these are termed kestotriose exohydrolases (Van den Ende et al., 2005), indicating that these forms might play a role in fructan signaling events (Van den Ende et al., 2004). It is known since long that FEHs also occur in non-fructan accumulators. However, they are probably better considered as "defective invertases" with possible (artificial) FEH side activities. The role of these proteins remained enigmatic for a very long period. However, a recent breakthrough paper (Le Roy et al., 2013) shows that Nin88, an apoplastic defective invertase from tobacco lacking FEH side activities, acts as indirect activator of active cell wall invertases (CWIs) which are crucial players in overall plant development, especially seed and fruit setting (Ruan et al., 2012). Although the exact underlying regulatory mechanisms require further research, data indicate that Nin88 interacts with cell walls in such a way that active CWIs bind to the cell wall in a more productive way (Le Roy et al., 2013). This fits

nicely within the emerging concept that dead enzymes are very common in all kingdoms of life and that many of them fulfil crucial biological roles, as reviewed in a recent Science paper (Leslie, 2013).

The first committed step in RFO biosynthesis is the production of galactinol (Gol) from *myo*-inositol and UDP-Gal, a reaction catalyzed by galactinol synthase (GolS; Keller and Pharr, 1996). Next, Gol is used as a donor to deliver Gal to Suc, creating Raf. This is catalyzed by raffinose synthase (RafS). Stachyose synthase uses Gol as donor and Raf as acceptor to synthesize Sta (Keller and Pharr, 1996). GolS, RafS, and StaS are believed to localize in the cytosol, although the RFOs they produce might also enter the vacuole and the chloroplasts (Nägele and Heyer, 2013). In some species, higher DP RFOs are produced by the action of galactan:galactan galactosyl transferases (GGTs; Bachmann et al., 1994), using RFOs as donor and acceptor substrates. Although the exact origin of manninotriose type of RFO in red deadnettle is not known, it was suggested that this compound results from invertase (β-fructosidase) activity on Sta (dos Santos et al., 2013). Lychnose synthase and stellariose synthase are the enzymes involved in the biosynthesis of lychnose and stellariose (Vanhaecke et al., 2010). RFO catabolism involves the activity of acid and alkaline α-galactosidases which sequentially remove the terminal Gal residues (Keller and Pharr, 1996), while β-fructosidases may produce melibiose (Galα1,6Glc) from Raf and manninotriose from Sta (dos Santos et al., 2013). The so-called seed imbibition proteins resemble the enzymes involved in RFO catabolism, but only a few forms have been functionally characterized (Peters et al., 2010). Similar to defective invertases, it can be speculated that some of these forms may represent catalytically inactive forms, acting as regulatory proteins. Some forms may be involved in the degradation of RFOs acting as cellular signals (see below).

ENZYMES: STRUCTURE–FUNCTION RELATIONSHIPS

The overall classification into families of carbohydrate active enzymes[1] is based on amino acid sequence similarities (Cantarel et al., 2009). This classification (i) reflects the structural features of these enzymes better than their sole substrate specificity, (ii) helps to reveal the evolutionary relationship between these enzymes, and (iii) provides a convenient framework to understand mechanistic properties (Henrissat and Romeu, 1995).

Plant acid invertases (β-fructosidases), including vacuolar invertases (VIs) and CWIs, split Suc into Fru and Glc by hydrolysis of the glycosidic bond. FEHs hydrolyze a terminal Fru from a fructan chain, while FTs elongate a Suc or fructan molecule with an extra Fru moiety. Taken together, all these enzymes transfer a Fru unit either to water (hydrolysis), to Suc or fructan (Van den Ende et al., 2009). They only differ in their specificity for donor and acceptor substrates. Accordingly, the 3D structure determinations of a FEH from chicory (Verhaest et al., 2005), a CWI from *Arabidopsis* (Lammens et al., 2008) and a pFT from *Pachysandra terminalis* (Lammens et al., 2012) showed that all these enzymes (or proteins: defective invertases) have a common fold. Hence, they are grouped together with microbial β-fructosidases (degrading both Suc and fructans) in the family 32 of glycoside hydrolases (GH32). Family GH32 is combined with family GH68 in the clan GH-J. GH68 harbors bacterial invertases, levansucrases and inulosucrases. All these proteins consist of an N-terminal five-bladed β-propeller domain (GH32 and GH68) followed by a C-terminal domain formed by two β-sheets (only in GH32). The active site is present within the β-propeller domain and characterized by the presence of three highly conserved acidic groups (present in the WMN**D**PNG, R**D**P, and **E**C motifs). The Asp from the first motif is acting as nucleophile, the Asp from the second motif is believed to be a transition state stabilizer and the Glu residue from the **E**C motif acts as acid/base catalyst playing a crucial role in the catalytic mechanism (Van den Ende et al., 2009). Some sugars can bind as substrates or as inhibitors in the active site of plant GH32 members (Verhaest et al., 2007) and this depends on subtle amino acid variations in the active site area. Recent pKa calculations suggest that most GH-J members show an acid–base catalyst that is not sufficiently protonated before ligand entrance, while the acid–base can be fully protonated when a substrate, but not an inhibitor, enters the catalytic pocket (Yuan et al., 2012). Moreover, the conserved arginine in the RDP motif, rather than a previously proposed Tyr in the FYASK motif, is proposed to play a key role to increase the pKa of the acid–base catalyst (Yuan et al., 2012).

Intriguingly, defective invertases are never affected in their catalytic triad, but rather in a neighboring "Asp/Lys" or "Asp/Arg couple" (present in a flexible loop in the proximity of the acid/base catalyst) and in some Trp residues (Le Roy et al., 2007, 2013). These residues are essential to stabilize the Glc part of Suc in the active site of GH32 Suc splitting enzymes (CWINV, VI, 1-SST, 6-SFT; Van den Ende et al., 2009), and they are absent in enzymes that use fructans as donor substrates (FEH, 1-FFT, 6G-FFT). This was confirmed by site directed mutagenesis experiments on invertase, defective invertase, FEH and 6G-FFT (Le Roy et al., 2007, 2008, 2013; Lasseur et al., 2009). However, the presence of an Asp/Lys or Asp/Arg is not sufficient; this couple needs to be in the right 3D configuration as well (Schroeven et al., 2009). The recent 3D structure of *Pachysandra terminalis* with its acceptor substrate 6-kestotriose strongly suggested that the couple (Asp/Gln in this case) plays a prominent role in acceptor substrate specificity as well (Lammens et al., 2012).

All RFO metabolizing enzymes discussed in the previous section, with the exception of GolS, belong to GH27 and GH36 in clan D. The acid α-galactosidases and GGTs are grouped into GH27, where some 3D structures have been determined, including the acid α-galactosidase from rice (Fujimoto et al., 2003). Their active sites are well-conserved and formed by residues in the loops at the ends of the β-strands in a $(\beta/\alpha)_8$ barrel. Two Asp residues are required for catalysis, which are positioned on opposite sides of the labile glycosidic bond (Fujimoto et al., 2003). RafS, StaS, and alkaline α-galactosidases belong to the related GH36, but no structural information is yet available on plant members within this family (Vanhaecke, 2010), although a few microbial structures became available (Fredslund et al., 2011; Merceron et al., 2012). To our knowledge, no in depth structure–function research has been performed toward donor and acceptor substrate specificities within plant members of GH27 and GH36. Clearly, such studies would be

[1] http://www.cazy.org/

very informative as well. Such insights greatly contribute to rational enzyme design contributing to the production of tailor-made fructans and RFOs.

EVOLUTION

Within GH32, it became clear that plant FTs evolved from VIs (Wei and Chatterton, 2001; Altenbach et al., 2009), contributing to the observed diversity in fructan accumulators in the plant kingdom (**Figure 1**). Two types of VIs (I and II, Van den Ende et al., 2002b) can be discerned in plants and for a long time it was assumed that all plant FTs evolved from (different forms of) type II VIs. This occurred at least three times: (i) in the Asterales (inulin-type of fructans; e.g., chicory, (ii) in the Poales with further distinction between cool-season grasses (mainly levan and neokestose-derived fructans, e.g., ryegrass) and cereals (predominantly graminan-type fructans, e.g., wheat and barley) in the Poaceae and (iii) in the Asparagales further splitting into the Allioideae (e.g., onion) and Agavoideae (e.g., Agave) subfamilies that also mainly accumulate neokestose-based fructans (**Figure 1**). However, this view was changed by the unexpected discovery of both levan- and graminan-type fructans in the basal eudicot *Pachysandra terminalis* species, containing a pFT that, surprisingly, evolved from a type I VI (**Figure 2**) and not from a type II VI as observed for all other FTs (**Figure 2**). This further confirmed the polyphyletic origin of fructan biosynthesis (Altenbach et al., 2009; Van den Ende et al., 2011a) and suggests that the capacity for fructan biosynthesis arose at least four times during the plant diversification process (**Figure 1**). Such polyphyletic origin did not likely occur within GH27 and GH36, although more sequences should be generated to reach this conclusion (Vanhaecke, 2010). By combining alignments, 3D structure information and phylogenetic analyses (Schroeven et al., 2008; Altenbach et al., 2009; Lasseur et al., 2011; Lammens et al., 2012), the current view within GH32 is that an ancestral VI duplicated in two VI types (I and II, **Figure 2**) before the separation of monocots and dicots (Wei and Chatterton, 2001). Most probably, monocot and dicot type II VIs were than recruited to create preliminary 1-SSTs and 6-SFTs that later specialized into genuine 1-SSTs and 6-SFTs (**Figure 2**). Mutations

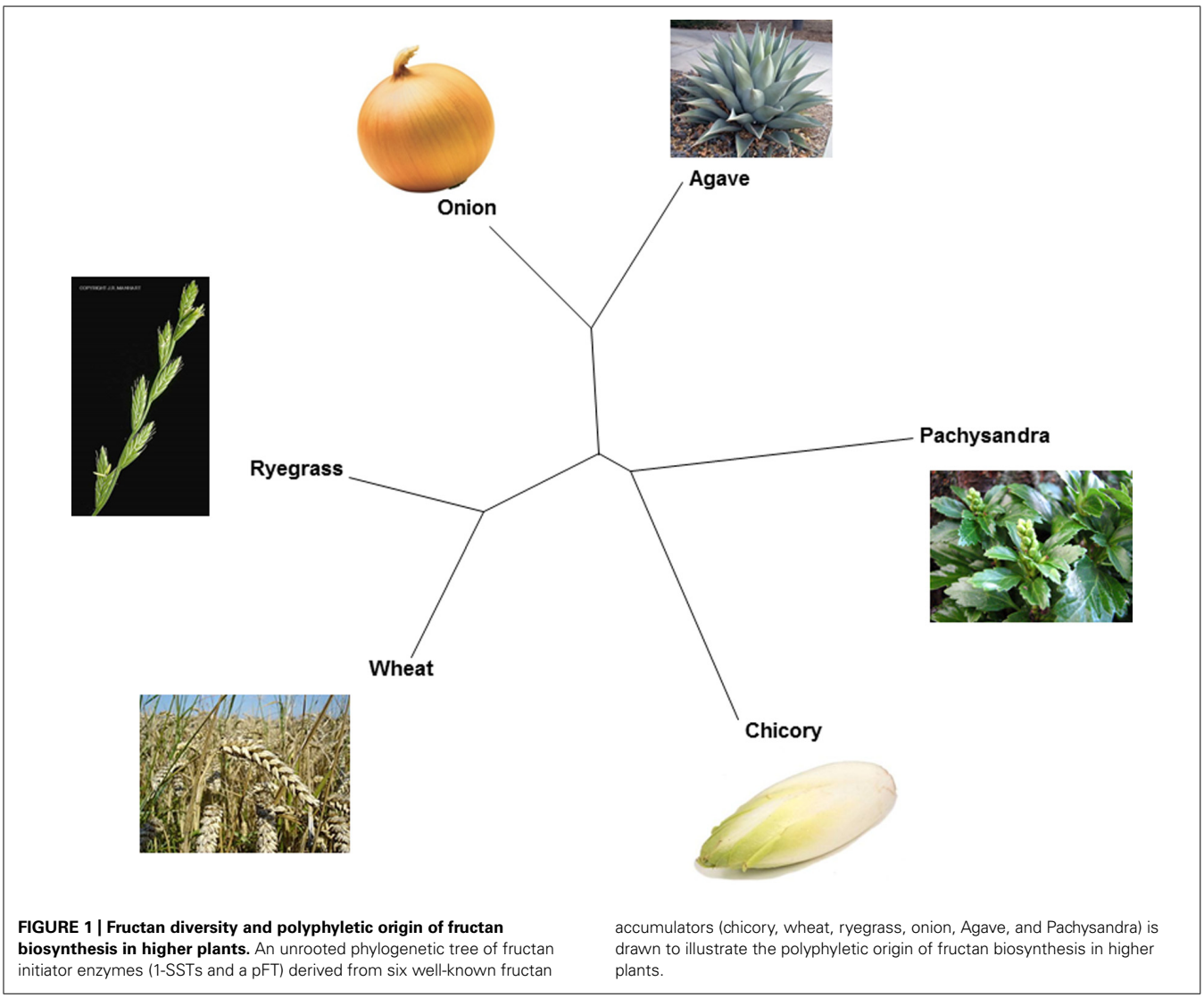

FIGURE 1 | Fructan diversity and polyphyletic origin of fructan biosynthesis in higher plants. An unrooted phylogenetic tree of fructan initiator enzymes (1-SSTs and a pFT) derived from six well-known fructan accumulators (chicory, wheat, ryegrass, onion, Agave, and Pachysandra) is drawn to illustrate the polyphyletic origin of fructan biosynthesis in higher plants.

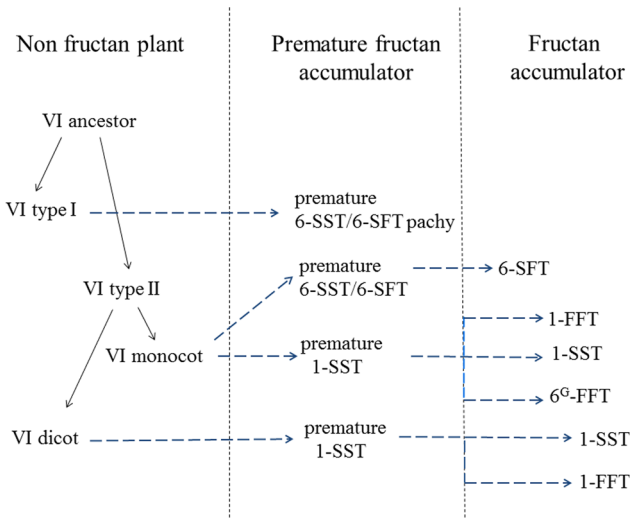

FIGURE 2 | Model depicting the putative evolution of different higher plant fructosyl transferases. Likely, an ancestral VI duplicated in two VI types (I and II) in a non-fructan-accumulating plant, probably before the separation of monocots and dicots. Within the basal eudicots, a type I VI was recruited to create a preliminary FT (a 6-SST/6-SFT) in *Pachysandra terminalis*, a recent evolutionary event. During older evolutionary events, certain monocot and dicot type II VIs evolved into preliminary 1-SSTs and 6-SST/6-SFTs that later specialized into genuine, more specific 1-SSTs and 6-SFTs. Likely, 1-FFTs (dicots) and 6G-FFTs (monocots) evolved later, based on (preliminary) 1-SST precursors. See text for more details.

in the "WMNDPNG" and "W(A/G)W" motifs are believed to play a key role in such processes (Schroeven et al., 2008, 2009; Altenbach et al., 2009). In evolutionary terms, it seems reasonable to assume that, in monocots as well as in dicots, 1-FFTs and 6G-FFTs evolved later, likely from (premature) 1-SST precursors (**Figure 2**). For instance, in wheat the identity between Ta1-SST and Ta1-FFT is much higher (84%) than between Ta1-SST and TaVI (67%) and between Ta1-FFT and TaVI (66%), strongly suggesting that Ta1-FFT evolved from Ta1-SST (**Figure 2**; Schroeven et al., 2009). A similar reasoning led to the hypothesis that the *Lolium perenne* Lp6^G-FFT evolved from a (premature) Lp1-SST (**Figure 2**; Lasseur et al., 2009). In the same way, it can be speculated that the chicory Ci1-FFT evolved from a (premature) Ci1-SST (**Figure 2**; Schroeven et al., 2009). Within the basal eudicots, a type I VI developed into a pFT in *Pachysandra terminalis* (**Figure 2**) and this is considered as a rather "recent" evolutionary event (Van den Ende et al., 2011a). On the contrary, defective invertases and FEHs evolved from CWIs within GH32 (Le Roy et al., 2007, 2013). It can be speculated that the loss or alteration of the above-mentioned "couple" is an early evolutionary event that led to the formation of defective invertases with cell wall localization and a high isoelectric point (pI) for interaction with the cell wall. To further develop genuine FEHs in fructan plants, it can be further hypothesized that precursor defective invertases retrieved (i) a vacuolar targeting signal for sorting to the central vacuole, (ii) a low pI typical for vacuolar proteins, (iii) amino acid alterations that helped stabilization of higher DP fructans as donor substrates (Le Roy et al., 2008).

CLASSIC FUNCTIONS OF FRUCTANS AND RFOs

The most widely accepted function of fructans is their role as a storage carbohydrate. Dicots typically store inulin-type fructans in underground reserve organs (roots, tubers) (Van Laere and Van den Ende, 2002) while monocots typically store fructans on a shorter term basis in above ground parts of the plant (Pollock and Cairns, 1991; Slewinski, 2012). To the best of our knowledge, fructans are the only neutral type of polysaccharides that accumulate in plant vacuoles. Fructans can accumulate to 20% on fresh weight basis and even up to 70% on a dry weight basis in some organs (Wiemken et al., 1995). Trying to solubilize such levels *in vitro* invariably leads to fructan precipitation, suggesting that fructans *in vivo* should be organized in a special way to keep them in a (semi)-soluble condition in the vacuole (Van den Ende, 1996). It is clear that starch is the most widespread reserve carbohydrate in the plant kingdom. On the one hand, insoluble starch granules represent a very elegant way of storing huge amounts of carbon in a very small volume. On the other hand, excessive amounts of water-insoluble starch would be physically destructive to the chloroplast, its site of synthesis and storage in leaves. Therefore, fructans may have some advantages as compared to starch. One of the arguments in favor of using fructans could be the fact that starch biosynthesis dramatically decreases when the temperature drops below 10°C, whereas fructan biosynthesis is much less sensitive to low temperatures (Pollock, 1986). Another difference between starch and fructans might include the speed of its breakdown and carbon remobilization. While a large array of different enzymes (dikinases, phosphatases, starch hydrolases) are necessary to release small sugars from a starch granule (Stitt and Zeeman, 2012), water-soluble fructans are expected to be degraded much quicker by the action of FEHs as a single enzyme type. In grasses fructans are mainly stored in the leaf bases and used for regrowth after defoliation (Morvan-Bertrand et al., 2001). In cereals, fructans temporarily accumulate in stems and early in seed development (Van den Ende et al., 2003; Van den Ende et al., 2011b; Joudi et al., 2012) as well as in reproductive organs (Ji et al., 2010). Contrary to the situation in dicots, where growth and fructan accumulation are usually separated in time, monocots are able to combine these processes. It could be argued that the activity of Suc splitting enzymes 1-SST and 6-SFT contribute to control and maintain sink strength and carbohydrate supplies (Ji et al., 2010), but then the obvious question can be raised why this is not simply accomplished by increasing the activity of invertases? This indicates that the accumulation of fructans as such should somehow be beneficial (see also below), especially under stress.

Fructans can also play a role during flower opening. Fructan contents are high in closed petals of *Campanula rapunculoides* and *Hemerocallis* while no fructan is present anymore in petals of opened flowers (Bieleski, 1993; Vergauwen et al., 2000). FEHs quickly release massive amounts of Fru, lowering the osmotic potential and contributing to water inflow and flower opening. Fructans appear to have additional functions in drought, salt, and freezing tolerance of plants (Valluru and Van den Ende, 2008; Livingston et al., 2009). This is further supported by the fact that fructan-accumulating plants are especially abundant in temperate and arid climate zones with seasonal frost or drought periods, and are almost absent in tropical regions (Hendry, 1993).

In fructan species, fructan accumulation can be induced under drought (De Roover et al., 2000) and cold (Livingston and Henson, 1998; Yoshida et al., 2007). More direct evidence comes from the observation that fructan-accumulating transgenic plants show enhanced stress tolerance (Pilon-Smits et al., 1995, 1999; Konstantinova et al., 2002; Li et al., 2007; Kawakami et al., 2008; Bie et al., 2012). Transgenic perennial ryegrass expressing wheat 1-SST or 6-SFT genes accumulate more fructans and acquired higher tolerance for freezing at the cellular level (Hisano et al., 2004). Therefore, it would be interesting to introduce FT genes in a number of food and biomass crops, to make them more tolerant to abiotic stresses.

Next to fructans, RFOs are also used as "storage carbohydrates," arbitrarily defined as those which occur at more than 1% of the dry weight of a given tissue. So, despite the fact that most plants synthesize RFOs (at least Raf) to some extent at some stage of their development, only some plants accumulate large amounts of them (Kandler and Hopf, 1984; Keller and Pharr, 1996). These RFO accumulators store RFOs in concentrations up to 25–80% of their dry weight in specialized storage organs such as tubers (e.g., *Stachys sieboldii*), seeds (e.g., soybean, lentil, chickpea), or in photosynthesizing leaves (e.g., *Ajuga reptans*; Bachmann et al., 1994; Tahir et al., 2012). Similar to fructans, and in contrast to starch, RFOs are osmotically flexible as their DP may easily change and so the osmotic pressure. Species that use Suc as reserve carbohydrate (sugar beet, sugar cane) can only double the osmotic pressure upon hydrolysis (Gilbert et al., 1997). Finally, RFOs are phloem-mobile, and are readily available for carbon translocation when required. This feature is less clear for fructans, since phloem mobility has only been documented in a single fructan accumulator (see below). Typically, strong RFO and fructan accumulation do not occur together in a single plant species, suggesting that RFOs and fructans might fulfill similar (or partially overlapping) physiological functions. To better understand subtle differences in their physiological functions, it would be interesting to seek for plants that are capable to store high levels of both RFOs and fructans. Similar to the introduction of FTs in non-fructan accumulators, the overexpression of GolS in *Arabidopsis thaliana* resulted in plants with increased Raf levels and increased stress tolerance (Taji et al., 2002; Nishizawa et al., 2008). This suggests that the presence of increased levels of fructans or RFOs (in plants that normally contain very low or undetectable levels of such components) helps plants to survive adverse climatic conditions.

MEMBRANE STABILIZATION AND ANTIOXIDANT PROPERTIES

What could be the underlying mechanisms to explain such increased stress tolerances? Since membranes (and critical membrane proteins) are one of the primary targets of freezing and desiccation injury in cells (Oliver et al., 2000), membrane protective effects have been dedicated to fructans as well as to RFOs. *In vitro* experiments provided evidence for this ability, demonstrating that both fructans and RFOs contribute to enhanced membrane stability during freezing and cellular dehydration by deep insertion between the headgroups of lipids, both in mono- and bilayers (Demel et al., 1998; Vereyken et al., 2001; Hincha et al., 2002, 2003; Valluru and Van den Ende, 2008; Valluru et al., 2008). As such, they are also well-positioned to scavenge hydroxyl radicals ($^{\cdot}OH$) which might originate from tonoplast-associated Class III peroxidase activities (Passardi et al., 2004; Van den Ende and Valluru, 2009). Among the biologically relevant reactive oxygen species (ROS: H_2O_2, $O_2^{\cdot-}$ and $^{\cdot}OH$), hydroxyl radicals are the most reactive and dangerous species (Keunen et al., 2013). The $^{\cdot}OH$ is known to react with almost all biomolecules at rates as those occurring in diffusion-controlled reactions (Hernandez-Marin and Martínez, 2012). As a consequence there are no enzymatic systems known to neutralize them in any living beings (Gechev et al., 2006). The *in vitro* $^{\cdot}OH$ scavenging activity of Raf and fructans has recently been confirmed (Stoyanova et al., 2011; Peshev et al., 2013) and compared to an array of phenolic compounds, well-known superior antioxidants (Peshev et al., 2013). Based on these findings, a hypothetical model has been proposed explaining how vacuolar fructans and phenolic compounds may act in a synergistic way to contribute to vacuolar antioxidant mechanisms *in vivo,* and to overall cellular homeostasis (Peshev et al., 2013). While fructans are obvious candidates for tonoplast stabilization and protection, RFOs (Raf in cold-induced *Arabidopsis* leaves) that are synthesized in the cytosol are candidates to protect the plasma membrane. However, this seems not to be the target membrane in *Arabidopsis* (Nägele and Heyer, 2013). Instead, it was demonstrated that Raf specifically acts to protect the photosystems located in the thylakoid membranes of plastids from damage during freeze thaw cycles (Knaupp et al., 2011). It was recently demonstrated that Raf can be imported in chloroplasts (Schneider and Keller, 2009) and therefore it could function as a cryoprotectant. As explained above for fructans or other osmolytes, it can be speculated that the $^{\cdot}OH$ scavenging capacity of Raf counteracts membrane and protein damage, contributing to thylakoid membrane stability and chloroplast integrity under stress (Doltchinkova et al., 2013). Likewise, targeting the synthesis of mannitol, another well-known $^{\cdot}OH$ scavenger (Stoyanova et al., 2011), to chloroplasts resulted in increased resistance to oxidative stress (Shen et al., 1997a,b), similar to what is observed in GolS overexpressors with their increased Raf levels (Nishizawa et al., 2008).

SIGNALING?

Nowadays, Glc, Fru, and Suc-specific signaling pathways have been elucidated in plants (Rolland et al., 2006; Cho and Yoo, 2011; Li et al., 2011), already suggesting that a signaling role for other types of small endogenous sugars should not be simply neglected. It seems that (a) Suc-specific signaling pathway(s) contributes to plant defense responses (Bolouri Moghaddam and Van den Ende, 2013a,b). Increased Suc levels typically lead to increased levels of fructans, RFOs and/or anthocyanins (Teng et al., 2005; Martínez-Noël et al., 2009, 2010; Nägele and Heyer, 2013), perhaps controlled by (a single) Suc-specific signaling pathway(s) (Bolouri Moghaddam and Van den Ende, 2013a).

Gol and Raf are now recognized as signaling molecules during biotic stress responses (Kim et al., 2008) and a similar role during abiotic stress responses has been suggested for RFOs (Valluru and Van den Ende, 2011; Eyles et al., 2013) and for fructans (Van den Ende et al., 2004). This led to the hypothesis that both RFOs and small fructans might act as endogenous, phloem-mobile stress signals. Indeed, small fructans have been detected in the phloem

sap of Agave (Wang and Nobel, 1998) and it was reported that the fructan 6-kestotriose is phloem-mobile when it is produced by yeast invertase expressed in companion cells (Zuther et al., 2004). According to this view, the small fructans 1-kestotriose[2] and its derivative inulobiose[3] have been recently detected at very low levels in *Arabidopsis*, widely known as a strict non-fructan accumulator. What could be the origin of these small fructans in healthy *Arabidopsis* tissues? The most straightforward explanation is that these fructans are produced by the activities of VIs (AtVI1 and AtVI2), since *Arabidopsis* is lacking genuine FTs. *Arabidopsis* VIs were isolated before and found to contain considerable FT activities when tested at high Suc levels (De Coninck et al., 2005). Thus, next to Suc signaling, RFO and fructan signaling concepts should not be neglected and become the subject of intensive investigations. Possibly, such signaling events form the basis of the so-called "sugar-based resistance" or "sweet immunity" concept (Gomez-Ariza et al., 2007; Bolouri Moghaddam and Van den Ende, 2012, 2013b) in plants, but perhaps also in animals (see below).

UNIVERSAL IMMUNOSTIMULATORS?

Interest in fructans and RFOs increased during the last decade due to their health-promoting effects, selectively stimulating beneficial bacteria, acting as prebiotics (Shoaf et al., 2006; Kolida et al., 2007). These effects may be indirectly mediated through their fermentation products, but direct effects should not be neglected (Van den Ende et al., 2011b; Di Bartolomeo et al., 2013). Inulin-type fructans and fructo-oligosaccharides are the most studied and widely applied prebiotics isolated from chicory roots, and added to a variety of food products (Roberfroid et al., 2010). However, attention is shifting to longer DP and branched-type fructans (e.g., wheat graminans and Agave fructans) and RFOs as superior prebiotics (Urias-Silvas et al., 2008; Vanhaecke, 2010; Casiraghi et al., 2011; Jenkins et al., 2011), since these may provide a better "protection" (lowered colon cancer risk) over the whole length of the colon (Di Bartolomeo et al., 2013). Besides their prebiotic characteristics, fructans and RFOs are also emerging as important immunostimulators in animals and humans (Hoentjen et al., 2005; Seifert and Watzl, 2007; Vos, 2008; Delgado et al., 2012; Lee et al., 2012), as they likely do in plants (Bolouri Moghaddam and Van den Ende, 2012, 2013b). Taken all together, it can be speculated that these oligosaccharides may be involved in universal antioxidant and immunostimulatory mechanisms in plants, animals, humans, and perhaps in all eukaryotic organisms, but this requires further investigations. Needless to say, understanding the underlying mechanisms could greatly contribute to disease prevention strategies, both in plants and in mammals (Van den Ende et al., 2011b; Bolouri Moghaddam and Van den Ende, 2012, 2013b; Di Bartolomeo et al., 2013).

[2]http://gmd.mpimp-golm.mpg.de/Analytes/5578B0AB-DAA3-4DC7-80A9-321065427188.aspx

[3]http://plantmetabolomics.vrac.iastate.edu/ver2/view/MetabolitePlot.php?MetaboliteID=PM_8_1234l

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REFERENCES

Altenbach, D., Rudino-Pinera, E., Olvera, C., Boller, T., Wiemken, A., and Ritsema, T. (2009). An acceptor-substrate binding site determining glycosyl transfer emerges from mutant analysis of a plant vacuolar invertase and a fructosyltransferase. *Plant Mol. Biol.* 69, 47–56. doi: 10.1007/s11103-008-9404-7

Bachmann, M., Matile, P., and Keller, F. (1994). Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Cold acclimation, translocation and sink to source transition – discovery of chain elongation enzyme. *Plant Physiol.* 105, 1335–1345. doi: 10.1104/pp.105.4.1335

Bieleski, R. L. (1993). Fructan hydrolysis drives petal expansion in the ephemeral daylily flower. *Plant Physiol.* 103, 213–219.

Bie, X., Wang, K., She, M., Du, L., Zhang, S., Li, J., et al. (2012). Combinational transformation of three wheat genes encoding fructan biosynthesis enzymes confers increased fructan content and tolerance to abiotic stresses in tobacco. *Plant Cell Rep.* 31, 2229–2238. doi: 10.1007/s00299-012-1332-y

Bolouri Moghaddam, M., and Van den Ende, W. (2012). Sugars and plant innate immunity. *J. Exp. Bot.* 63, 3989–3998. doi: 10.1093/jxb/ers129

Bolouri Moghaddam, M., and Van den Ende, W. (2013a). Sugars, the clock and transition to flowering. *Front. Plant Sci.* 4:22. doi: 10.3389/fpls.2013.00022

Bolouri Moghaddam, M., and Van den Ende, W. (2013b). Sweet immunity in the plant circadian regulatory network. *J. Exp. Bot.* 64, 1439–1449. doi: 10.1093/jxb/ert046

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Casiraghi, M. C., Zanchi, R., Canzi, E., Pagani, M. A., Viaro, T., Benini, L., et al. (2011). Prebiotic potential and gastrointestinal effects of immature wheat grain (IWG) biscuits. *Antonie Van Leeuwenhoek* 99, 795–805. doi: 10.1007/s10482-011-9553-6

Chatterton, N. J., and Harrison, P. A. (1997). Fructan oligomers in *Poa ampla*. *New Phytol.* 136, 3–10. doi: 10.1046/j.1469-8137.1997.00716.x

Chatterton, N. J., Harrison, P. A., Thornley, W. R., and Bennett, J. H. (1993). Structures of fructan oligomers in orchardgrass (*Dactylis glomerata* L.). *J. Plant Physiol.*142, 552–556. doi: 10.1016/S0176-1617(11)80397-4

Cho, Y. H., and Yoo, S. D. (2011). Signaling role of fructose mediated by FINS1/FBP in *Arabidopsis thaliana*. *Plos Genetics* 7:e1001263. doi: 10.1371/journal.pgen.1001263

De Coninck, B., Le Roy, K., Francis, I., Clerens, S., Vergauwen, R., Halliday, A., et al. (2005). *Arabidopsis* AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. *Plant Cell Environ.* 28, 432–443. doi: 10.1111/j.1365-3040.2004.01281.x

Delgado, G. T. C., Thome, R., Gabriel, D. L., Tamashiro, W., and Pastore, G. M. (2012). Yacon (*Smallanthus sonchifolius*)-derived fructooligosaccharides improves the immune parameters in the mouse. *Nutr. Res.* 32, 884–892. doi: 10.1016/j.nutres.2012.09.012

Demel, R. A., Dorrepaal, E., Ebskamp, M. J. M., Smeekens, J. C. M., and de Kruijff, B. (1998). Fructans interact strongly with model membranes. *Biochim. Biophys. Acta* 1375, 36–42. doi: 10.1016/S0005-2736(98)00138-2

De Roover, L., Vandenbranden, K., Van Laere, A., and Van den Ende, W. (2000). Drought induces fructan synthesis and 1-SST (sucrose:sucrose fructosyltransferase) in roots and leaves of *Cichorium* seedlings (*Cichorium intybus* L.). *Planta* 210, 808–814. doi: 10.1007/s004250050683

Di Bartolomeo, F., Startek, J., and Van den Ende, W. (2013). Prebiotics to fight diseases: reality or fiction? *Phytother. Res.* doi: 10.1002/ptr.4901 [Epub ahead of print].

Dobrenel, T., Marchive, C., Azzopardi, M., Clément, G., Moreau, M., Sormani, R., et al. (2013). Sugar metabolism and the plant target of rapamycin kinase: a sweet operaTOR? *Front. Plant Sci.* 4:93. doi: 10.3389/fpls.2013.00093

Doltchinkova, V., Angelova, P., Ivanova, E., Djilianov, D., and Moyankova, D. (2013). Surface electric charge of thylakoid membranes from genetically modified tobacco plants under freezing stress. *J. Photochem.*

Photobiol. B. 119, 22–30. doi: 10.1016/j.jphotobiol.2012.12.004

dos Santos, R., Vergauwen, R., Pacolet, P., Lescrinier, E., and Van den Ende, W. (2013). Manninotriose is a major carbohydrate in red deadnettle (*Lamium purpureum*, Lamiaceae). *Ann. Bot.* 111, 385–393. doi: 10.1093/aob/mcs288

Edelman, J., and Jefford, T. G. (1968). The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*. *New Phytol.* 67, 517–531. doi: 10.1111/j.1469-8137.1968.tb05480.x

Eyles, A., Pinkard, E. A., Noel, W., Davies, N. W., Corkrey, R., Churchill, K., et al. (2013). Whole-plant- versus leaf-level regulation of photosynthetic responses after partial defoliation in *Eucalyptus globulus* saplings. *J. Exp. Bot.* 64, 1625–1636. doi: 10.1093/jxb/ert017

Fredslund, F., Hachem, M. A., Larsen, R. J., Sørensen, P. G., Coutinho, P. M., Lo Leggio, L., et al. (2011). Crystal structure of α-galactosidase from *Lactobacillus acidophilus* NCFM: insight into tetramer formation and substrate binding. *J. Mol. Biol.* 412, 466–480. doi: 10.1016/j.jmb.2011.07.057

Fujimoto, Z., Kaneko, S., Momma, M., Kobayashi, H., and Mizuno, H. (2003). Crystal structure of rice α-galactosidase complexed with D-galactose. *J. Biol. Chem.* 278, 20313–20318. doi: 10.1074/jbc.M302292200

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., and Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* 28, 1091–1101. doi: 10.1002/bies.20493

Gilbert, G. A., Wilson, C., and Madore, M. A. (1997). Root-zone salinity alters raffinose oligosaccharide metabolism and transport in Coleus. *Plant Physiol.* 115, 1267–1276. doi: 10.1104/pp.115.3.1267

Gomez-Ariza, J., Campo, S., Rufat, M., Estopa, M., Messeguer, J., San Segundo, B., et al. (2007). Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. *Mol. Plant Microbe Interact.* 20, 832–842. doi: 10.1094/MPMI-20-7-0832

Haritatos, E., Keller, F., and Turgeon, R. (1996). Raffinose oligosaccharide concentrations measured in individual cells and tissue types in *Cucumis melo* L. leaves: implications for phloem loading. *Planta* 198, 614–622. doi: 10.1007/BF00262649

Hendry, G. A. F. (1993). Evolutionary origins and natural functions of fructans - a climatological, biogeographic and mechanistic appraisal. *New Phytol.* 123, 3–14. doi: 10.1111/j.1469-8137.1993.tb04525.x

Henrissat, B., and Romeu, A. (1995). Families, superfamilies and subfamilies of glycosyl hydrolases. *Biochem. J.* 311, 350–351. doi: 10.1186/1471-2164-6-112

Hernandez-Marin, E., and Martínez, A. (2012). Carbohydrates and their free radical scavenging capability: a theoretical study. *J. Phys. Chem. B.* 116, 9668–9675. doi: 10.1021/jp304814r

Hincha, D. K., Zuther, E., Hellwege, E. M., and Heyer, A. G. (2002). Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology* 12, 103–110. doi: 10.1093/glycob/12.2.103

Hincha, D. K., Zuther, E., and Heyer, A. G. (2003). The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochim. Biophys. Acta* 1612, 172–177. doi: 10.1016/S0005-2736(03)00116-0

Hisano, H., Kanazawa, A., Kawakami, A., Yoshida, M., Shimamoto, Y., and Yamada, T. (2004). Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquired increased tolerance on a cellular level to freezing. *Plant Sci.* 167, 861–868. doi: 10.1016/j.plantsci.2004.05.037

Hoentjen, F., Welling, G. W., Harmsen, H. J., Zhang, X., Snart, J., Tannock, G. W., et al. (2005). Reduction of colitis by prebiotics in HLA-B27 transgenic rats is associated with microflora changes and immunomodulation. *Inflamm. Bowel Dis.* 11, 977–985. doi: 10.1097/01.MIB.0000183421.02316.d5

Hoffmann-Thoma, G., van Bel, A. J. E., and Ehlers, K. (1996). Ultrastructure of minor-vein phloem and assimilate transport in summer and winter leaves of the symplasmically loading evergreens *Ajuga reptans* L., *Aucuba japonica* Thunb., and *Hedera helix* L. *Planta* 212, 231–242. doi: 10.1007/s004250000382

Jenkins, C. L. D., Lewis, D., Bushell, R., Belobrajdic, D. P., and Bird, A. R. (2011). Chain length of cereal fructans isolated from wheat stem and barley grain modulates in vitro fermentation. *J. Cereal Sci.* 53, 188–191. doi: 10.1016/j.jcs.2010.12.001

Ji, X., Shiran, B., Wan, J., Lewis, D. C., Jenkins, C. L. D., Condon, A. G., et al. (2010). Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. *Plant Cell Environ.* 33, 926–942. doi: 10.1111/j.1365-3040.2010.02130.x

Joudi, M., Ahmadi, A., Mohamadi, V., Abbasi, A., Vergauwen, R., Mohamadi, H., et al. (2012). Comparison of fructan dynamics in two wheat cultivars with different capacities of accumulation and remobilizationunder terminal drought stress. *Physiol. Plant.* 144, 1–12. doi: 10.1111/j.1399-3054.2011.01517.x

Kandler, O., and Hopf, H. (1984). "Biosynthesis of oligosaccharides in vascular plants," in *Storage Carbohydrates in Vascular Plants*, ed. D. H. Lewis (Cambridge: Cambridge University Press), 115–132.

Kawakami, A., Sato, Y., and Yoshida, M. (2008). Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. *J. Exp. Bot.* 59, 803–814. doi: 10.1093/jxb/erm367

Keller, F., and Pharr, D. M. (1996). "Metabolism of carbohydrates in sinks and sources: galactosyl-sucrose oligosaccharides," in *Photoassimilate Distribution in Plants and Crops: Source–Sink Relationships*, eds E. Zamski and A. A. Schaffer (New York: Marcel Dekker), 157–183.

Keunen, E., Peshev, D., Vangronsveld, J., Van den Ende, W., and Cuypers, A. (2013). Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant Cell Environ.* doi: 10.1111/pce.12061

Kim, M. S., Cho, S. M., Kang, E. Y., Im, Y. J., Hwangbo, H., Kim, Y. C., et al. (2008). Galactinol is a signaling component of the induced systemic resistance caused by *Pseudomonas chlororaphis* O6 root colonization. *Mol. Plant Microbe Interact.* 21, 1643–1653. doi: 10.1094/MPMI-21-12-1643

Knaupp, M., Mishra, K. B., Nedbal, L., and Heyer, A. G. (2011). Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. *Planta* 234, 477–486. doi: 10.1007/s00425-011-1413-0

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Curr. Opin. Plant Biol.* 7, 235–246. doi: 10.1016/j.pbi.2004.03.014

Kolida, S., Meyer, D., and Gibson, G. R. (2007). A double-blind placebo-controlled study to establish the bifidogenic dose of inulin in healthy humans. *Eur. J. Clin. Nutr.* 61, 1189–1195. doi: 10.1038/sj.ejcn.1602636

Konstantinova, T., Parvanova, D., Atanassov, A., and Djilianov, D. (2002). Freezing tolerant tobacco, transformed to accumulate osmoprotectants. *Plant Sci.* 163, 157–164. doi: 10.1016/S0168-9452(02)00090-0

Lammens, W., Le Roy, K., Van Laere, A., Rabijns, A., and Van den Ende, W. (2008). Crystal structures of *Arabidopsis thaliana* cell-wall invertase mutants in complex with sucrose. *J. Mol. Biol.* 377, 378–385. doi: 10.1016/j.jmb.2007.12.074

Lammens, W., Le Roy, K., Yuan, S., Vergauwen, R., Rabijns, A., Van Laere, A., et al. (2012). Crystal structure of 6-SST/6-SFT from *Pachysandra terminalis*, a plant fructan biosynthesizing enzyme in complex with its acceptor substrate 6-kestose. *Plant J.* 70, 205–219. doi: 10.1111/j.1365-313X.2011.04858.x

Lasseur, B., Lothier, J., Wiemken, A., Van Laere, A., Morvan-Bertrand, A., Van den Ende, W., et al. (2011). Towards a better understanding of the generation of fructan structure diversity in plants: molecular and functional characterization of a sucrose:fructan 6-fructosyltransferase (6-SFT) cDNA from perennial ryegrass (*Lolium perenne*). *J. Exp. Bot.* 62, 1871–1885. doi: 10.1093/jxb/erq388

Lasseur, B., Schroeven, L., Lammens, W., Le Roy, K., Spangenberg, G., Manduzio, H., et al. (2009). Transforming a fructan:fructan 6G-fructosyltransferase from perennial ryegrass into a sucrose:sucrose 1-fructosyltransferase. *Plant Physiol.* 149, 327–339. doi: 10.1104/pp.108.125559

Lee, J. B., Miyake, S., Umetsu, R., Hayashi, K., Chijimatsu, T., and Hayashi, T. (2012). Anti-influenza A virus effects of fructan from Welsh onion (*Allium fistulosum* L.). *Food Chem.* 134, 2164–2168. doi: 10.1016/j.foodchem.2012.04.016

Le Roy, K., Lammens, W., Van Laere, A., and Van den Ende, W. (2008). Influencing the binding configuration of sucrose in the active sites of chicory fructan 1-exohydrolase and sugar beet fructan 6-exohydrolase. *New Phytol.* 178, 572–580. doi: 10.1111/j.1469-8137.2008.02386.x

Le Roy, K., Lammens, W., Verhaest, M., De Coninck, B., Rabijns, A., Van Laere, A., et al. (2007). Unraveling the difference between invertases and fructan exohydrolases: a single amino acid (Asp-239) substitution transforms *Arabidopsis* cell wall invertase1 into a fructan 1-exohydrolase. *Plant Physiol.* 145, 616–625. doi: 10.1104/pp.107.105049

Le Roy, K., Vergauwen, R., Struyf, T., Yuan, S., Lammens, W., Matrai, J., et al. (2013). Understanding the role of defective invertases in plants: tobacco Nin88 fails to degrade sucrose. *Plant Physiol.* 161, 1670–1681. doi: 10.1104/pp.112.209460

Leslie, M. (2013). 'Dead' enzymes show signs of life. *Science* 340, 25–27. doi: 10.1126/science.340.6128.25

Li, H.-J., Yang, A.-F., Zhang, X.-C., Gao, F., and Zhang, J.-R. (2007). Improving freezing tolerance of transgenic tobacco expressing-sucrose: sucrose 1-fructosyltransferase gene from *Lactuca sativa*. *Plant Cell Tiss. Org. Cult.* 89, 37–48. doi: 10.1007/s11240-007-9213-8

Li, P., Wind, J. J., Shi, X. L., Zhang, H. L., Hanson, J., Smeekens, S. C., et al. (2011). Fructose sensitivity is suppressed in *Arabidopsis* by the transcription factor ANAC089 lacking the membrane-bound domain. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3436–3441. doi: 10.1073/pnas.1018665108

Livingston, D. P. III, and Henson, C. A. (1998). Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: responses to second-phase cold hardening. *Plant Physiol.* 116, 403–408. doi: 10.1104/pp.116.1.403

Livingston, D. P. III, Hincha, D. K., and Heyer, A. G. (2009). Fructan and its relationship to abiotic stress tolerance in plants. *Cell. Mol. Life Sci.* 66, 2007–2023. doi: 10.1007/s00018-009-0002-x

Lu, C., Koroleva, O. A., Farrar, J. F., Gallagher, J., Pollock, C. J., and Tomos, A. D. (2002). Rubisco small subunit, chlorophyll a/b-binding protein and sucrose:fructan-6-fructosyl transferase gene expression and sugar status in single barley leaf cells in situ. Cell type specificity and induction by light. *Plant Physiol.* 130, 1335–1348. doi: 10.1104/pp.008979

Maleux, K., and Van den Ende, W. (2007). Levans in excised leaves of *Dactylis glomerata*: effects of light, sugars, temperature and senescence. *J. Plant Biol.* 50, 671–680. doi: 10.1007/BF03030612

Martínez-Noël, G. A., Tognetti, J. A., Salerno, G. L., and Pontis, H. G. (2010). Sugar signaling of fructan metabolism: new insights on protein phosphatases in sucrose-fed wheat leaves. *Plant Signal. Behav.* 5, 311–313. doi: 10.1007/s00425-009-1002-7

Martínez-Noël, G. M. A., Tognetti, J. A., Salerno, G. L., Wiemken, A., and Pontis, H. G. (2009). Protein phosphatase activity and sucrose–mediated induction of fructan synthesis in wheat. *Planta* 230, 1071–1079. doi: 10.1007/s00425-009-1002-7

Merceron, R., Foucault, M., Haser, R., Mattes, R., Watzlawick, H., and Gouet, P. (2012). The molecular mechanism of thermostable α-galactosidases AgaA and AgaB explained by X-ray crystallography and mutational studies. *J. Biol. Chem.* 287, 39642–39652. doi: 10.1074/jbc.M112.394114

Morvan-Bertrand, A., Boucaud, J., Le Saos, J., and Prud'homme, M. P. (2001). Roles of the fructans from leaf sheaths and from the elongating leaf bases in the regrowth following defoliation of *Lolium perenne* L. *Planta* 213, 109–120. doi: 10.1007/s004250000478

Nägele, T., and Heyer, A. G. (2013). Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana*. *New Phytol.* 198, 777–787. doi: 10.1111/nph.12201

Nishizawa, A., Yabuta, Y., and Shigeoka, S. (2008). Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.* 147, 1251–1263. doi: 10.1104/pp.108.122465

Ogawa, D., Yamaguchi, K., and Nishiuchi, T. (2007). High-level over expression of the *Arabidopsis* HsfA2 gene confers not only increased thermo tolerance but also salt/osmotic stress tolerance and enhanced callus growth. *J. Exp. Bot.* 58, 3373–3383. doi: 10.1093/jxb/erm184

Oliver, M. J., Tuba, Z., and Mishler, B. D. (2000). The evolution of vegetative desiccation tolerance in land plants. *Plant Ecol.* 151, 85–100. doi: 10.1023/A:1026550808557

Panikulangara, T. J., Eggers-Schumacher, G., Wunderlich, W., Stransky, H., and Schöffl, F. (2004). Galactinol synthase1. A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in *Arabidopsis*. *Plant Physiol.* 136, 3148–3158. doi: 10.1104/pp.104.042606

Passardi, F., Penel, C., and Dunand, C. (2004). Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci.* 9, 534–540. doi: 10.1016/j.tplants.2004.09.002

Pavis, N., Chatterton, N. J., Harrison, P. A., Baumgartner, S., Praznik, W., Boucaud, J., et al. (2001). Structure of fructans in roots and leaf tissues of *Lolium perenne*. *New Phytol.* 150, 83–95. doi: 10.1046/j.1469-8137.2001.00069.x

Peshev, D., Vergauwen, R., Moglia, A., Hideg, E., and Van den Ende, W. (2013). Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *J. Exp. Bot.* 64, 1025–1038. doi: 10.1093/jxb/ers377

Peters, S., Egert, A., Stieger, B., and Keller, F. (2010). Functional identification of *Arabidopsis* ATSIP2 (At3g57520) as an alkaline α-galactosidase with a substrate specificity for raffinose and an apparent sink-specific expression pattern. *Plant Cell Physiol.* 51, 1815–1819. doi: 10.1093/pcp/pcq127

Pilon-Smits, E. A. H., Ebskamp, M. J. M., Paul, M. J., Jeuken, M. J. W., Weisbeek, P. J., and Smeekens, S. C. M. (1995). Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol.* 107, 125–130. doi: 10.1104/pp.107.1.125

Pilon-Smits, E. A. H., Terry, N., Sears, T., and van Dun, K. (1999). Enhanced drought resistance in fructan-producing sugar beet. *Plant Physiol. Biochem.* 37, 313–317. doi: 10.1016/S0981-9428(99)80030-8

Pollock, C. J. (1986). Fructans and the metabolism of sucrose in vascular plants. *New Phytol.* 104, 1–24. doi: 10.1111/j.1469-8137.1986.tb00629.x

Pollock, C. J., and Cairns, A. J. (1991). Fructan metabolism in grasses and cereals. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 42, 77–101. doi: 10.1146/annurev.pp.42.060191.000453

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., et al. (2010). Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 104, S1–S63. doi: 10.1017/S0007114510003363

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Ann. Rev. Plant Biol.* 57, 675–709. doi: 10.1146/annurev.arplant.57.032905.105441

Ruan, Y.-L., Patrick, J. W., Bouzayen, M., Osorio, S., and Fernie, A. R. (2012). Molecular regulation of seed and fruit set. *Trends Plant Sci.* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Schneider, T., and Keller, F. (2009). Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. *Plant Cell Physiol.* 50, 2174–2182. doi: 10.1093/pcp/pcp151

Schroeven, L., Lammens, W., Kawakami, A., Yoshida, M., Van Laere, A., and Van den Ende, W. (2009). Creating S-type characteristics in the F-type enzyme fructan:fructan 1-fructosyltransferase of *Triticum aestivum* L. *J. Exp. Bot.* 60, 3687–3696. doi: 10.1093/jxb/erp208

Schroeven, L., Lammens, W., Van Laere, A., and Van den Ende, W. (2008). Transforming wheat vacuolar invertase into a high affinity sucrose: sucrose 1-fructosyltransferase. *New Phytol.* 180, 822–831. doi: 10.1111/j.1469-8137.2008.02603.x

Seifert, S., and Watzl, B. (2007). Inulin and oligofructose: review of experimental data on immune modulation. *J. Nutr.* 137, 2563S–2567S. doi: 10.1038/ejcn.2009.64

Shen, B., Jensen, R. C., and Bohnert, H. J. (1997a). Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. *Plant Physiol.* 113, 1177–1183. doi: 10.1104/pp.113.4.1177

Shen, B., Jensen, R. C., and Bohnert, H. J. (1997b). Mannitol protects against oxidation by hydroxyl radicals. *Plant Physiol.* 115, 527–532. doi: 10.1104/pp.115.2.527

Shoaf, K., Mulvey, G. L., Armstrong, G. D., and Hutkins, R. W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect. Immun.* 74, 6920–6928. doi: 10.1128/IAI.01030-06

Slewinski, T. L. (2012). Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. *J. Exp. Bot.* 63, 4647–4670. doi: 10.1093/jxb/ers124

Stitt, M., and Zeeman, S. C. (2012). Starch turnover: pathways, regulation and role in growth. *Curr. Opin. Plant Biol.* 15, 282–292. doi: 10.1016/j.pbi.2012.03.016

Stoyanova, S., Geuns, J., Hideg, E., and Van den Ende, W. (2011). The food additives inulin and stevioside

counteract oxidative stress. *Int. J. Food Sci. Nutr.* 62, 207–214. doi: 10.3109/09637486.2010.523416

Tahir, M., Baga, M., Vandenberg, A., and Chibbar, R. N. (2012). An assessment of raffinose family oligosaccharides and sucrose concentration in genus *Lens*. *Crop Sci.* 52, 1713–1720. doi: 10.2135/cropsci2011.08.0447

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., et al. (2002). Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.* 29, 417–426. doi: 10.1046/j.0960-7412.2001.01227.x

Tamura, K., Kawakami, A., Sanada, Y., Tase, K., Komatsu, T., and Yoshida, M. (2009). Cloning and functional analysis of a fructosyltransferase cDNA for synthesis of highly polymerized levans in timothy (*Phleum pratense* L.). *J. Exp. Bot.* 60, 893–905. doi: 10.1093/jxb/ern337

Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M., and Smeekens, S. (2005). Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires the MYB75/PAP1 gene. *Plant Physiol.* 139, 1840–1852. doi: 10.1104/pp.105.066688

Turgeon, R., Medville, R., and Nixon, K. C. (2001). The evolution of minor veinphloem and phloem loading. *Am. J. Bot.* 88, 1331–1339. doi: 10.2307/3558441

Urias-Silvas, J. E., Cani, P. D., Delmee, E., Neyrinck, A., Lopez, M. G., and Delzenne, N. M. (2008). Physiological effects of dietary fructans extracted from *Agave tequilana* Gto. *and Dasylirion* spp. *Br. J. Nutr.* 99, 254–261. doi: 10.1017/S0007114507795338

Valluru, R., Lammens, W., Claupein, W., and Van den Ende, W. (2008). Freezing tolerance by vesicle-mediated fructan transport. *Trends Plant Sci.* 13, 409–414. doi: 10.1016/j.tplants.2008.05.008

Valluru, R., and Van den Ende, W. (2008). Plant fructans in stress environments: emerging concepts and future prospects. *J. Exp. Bot.* 59, 2905–2916. doi: 10.1093/jxb/ern164

Valluru, R., and Van den Ende, W. (2011). Myoinositol and beyond – emerging networks under stress. *Plant Sci.* 181, 387–400. doi: 10.1016/j.plantsci.2011.07.009

Van den Ende, W. (1996). *Fructan Metabolism in Chicory Roots* (*Cichorium intybus* L.). Ph.D. thesis, KU Leuven, Belgium.

Van den Ende, W., Clerens, S., Vergauwen, R., Van Riet, L., Van Laere, A., Yoshida, M., et al. (2003). Fructan 1-exohydrolases. β (2,1) trimmers during graminan biosynthesis in stems of wheat (*Triticum aestivum* L.)? purification, characterization, mass mapping and cloning of two fructan 1-exohydrolase isoforms. *Plant Physiol.* 131, 621–631. doi: 10.1104/pp.015305

Van den Ende, W., Coopman, M., Clerens, S., Vergauwen, R., Le Roy, K., Lammens, W., et al. (2011a). Unexpected presence of graminan- and levan-type fructans in the evergreen frost-hardy eudicot *Pachysandra terminalis* (*Buxaceae*). Purification, cloning and functional analysis of a 6-SST/6-SFT enzyme. *Plant Physiol.* 155, 603–614. doi: 10.1104/pp.110.162222

Van den Ende, W., Peshev, D., and De Gara, L. (2011b). Disease prevention by natural antioxidants and prebiotics acting as ROS scavengers in the gastrointestinal tract. *Trends Food Sci. Tech.* 22, 689–697. doi: 10.1016/j.tifs.2011.07.005

Van den Ende, W., De Coninck, B., and Van Laere, A. (2004). Plant fructan exohydrolases: a role in signaling and defense? *Trends Plant Sci.* 9, 523–528. doi: 10.1016/j.tplants.2004.09.008

Van den Ende, W., De Roover, J., and Van Laere, A. (1996). In vitro synthesis of fructofuranosylonlyoligosaccharides from inulin and fructose by purified chicory root fructan:fructan fructosyl transferase. *Physiol. Plant.* 97, 346–352. doi: 10.1034/j.1399-3054.1996.970219.x

Van den Ende, W., Lammens, W., Van Laere, A., Schroeven, L., and Le Roy, K. (2009). Donor and acceptor substrate selectivity among plant glycoside hydrolase family 32 enzymes. *FEBS J.* 276, 5788–5798. doi: 10.1111/j.1742-4658.2009.07316.x

Van den Ende, W., Michiels, A., De Roover, J., and Van Laere, A. (2002a). Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases. Expression of fructan genes throughout chicory development. *ScientificWorldJoural* 2, 1273–1287. doi: 10.1100/tsw.2002.288

Van den Ende, W., Michiels, A., Le Roy, K., and Van Laere, A. (2002b). Cloning of a vacuolar invertase from Belgian endive leaves (*Cichorium intybus* L.). *Physiol. Plant.* 115, 504–512. doi: 10.1034/j.1399-3054.2002.1150404.x

Van den Ende, W., and Valluru, R. (2009). Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? *J. Exp. Bot.* 60, 9–18. doi: 10.1093/jxb/ern297

Van den Ende, W., Yoshida, M., Clerens, S., Vergauwen, R., and Kawakami, A. (2005). Cloning, characterization and functional analysis of novel 6-kestose exohydrolases (6-KEHs) from wheat (*Triticum aestivum*). *New Phytol.* 166, 917–932. doi: 10.1111/j.1469-8137.2005.01394.x

Vanhaecke, M. (2010). *Metabolism of Galactosyl-Oligosaccharides in Caryophyllaceae*. Ph.D. thesis, Katholieke Universiteit Leuven, Belgium.

Vanhaecke, M., Dyubankova, N., Lescrinier, E., and Van den Ende, W. (2010). Metabolism of galactosyl-oligosaccharides in *Stellaria media* - discovery of stellariose synthase, a novel type of galactosyltransferase. *Phytochem.* 71, 1095–1103. doi: 10.1016/j.phytochem.2010.04.012

Vanhaecke, M., Van den Ende, W., Lescrinier, E., and Dyubankova, N. (2008). Isolation and characterization of a pentasaccharide from *Stellaria media*. *J. Nat. Prod.* 71, 1833–1836. doi: 10.1021/np800274k

Vanhaecke, M., Van den Ende, W., Van Laere, A., Herdewijn, P., and Lescrinier, E. (2006). Complete NMR characterization of lychnose from *Stellaria media* (L.) Vill. *Carbohydr. Res.* 341, 2744–2750. doi: 10.1016/j.carres.2006.09.001

Van Laere, A., and Van den Ende, W. (2002). Inulin metabolism in dicots: chicory as a model system. *Plant Cell Environ.* 25, 803–813. doi: 10.1046/j.1365-3040.2002.00865.x

Vereyken, I. J., Chupin, V., Demel, R. A., Smeekens, S. C. M., and De Kruijff, B. (2001). Fructans insert between the headgroups of phospholipids. *Biochim. Biophys. Acta Biomem.* 1510, 307–320. doi: 10.1016/S0005-2736(00)00363-1

Vergauwen, R., Van den Ende, W., and Van Laere, A. (2000). The role of fructan in flowering of *Campanula rapunculoides*. *J. Exp. Bot.* 51, 1261–1266. doi: 10.1093/jexbot/51.348.1261

Verhaest, M., Lammens, W., Le Roy, K., De Ranter, C., Van Laere, A., Rabijns, A., et al. (2007). Insights into the fine architecture of the active site of chicory fructan 1- exohydrolase: 1-kestose as substrate vs sucrose as inhibitor. *New Phytol.* 174, 90–100. doi: 10.1111/j.1469-8137.2007.01988.x

Verhaest, M., Van den Ende, W., Le Roy, K., De Ranter, C. A., Van Laere, A., and Rabijns, A. (2005). X-Ray diffraction structure of a plant glycosyl hydrolase family 32 protein: fructan 1-exohydrolase IIa of *Cichorium intybus*. *Plant J.* 41, 400–411. doi: 10.1111/j.1365-313X.2004.02304.x

Vijn, I., and Smeekens, S. (1999). Fructan: more than a reserve carbohydrate? *Plant Physiol.* 120, 351–359. doi: 10.1104/pp.120.2.351

Vos, A. J. (2008). *Preclinical Studies on the Immune-Modulatory Effects of Dietary Oligosaccharides*. Ph.D. thesis, Maastricht University, Netherlands.

Wang, N., and Nobel, P. S. (1998). Phloem transport of fructans in the crassulacean acid metabolism species *Agave deserti*. *Plant Physiol.* 116, 709–714. doi: 10.1104/pp.116.2.709

Wang, Z., Zhu, Y., Wang, L., Liu, X., Liu, Y., Phillips, J., et al. (2009). A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (BhGolS1) promoter. *Planta* 230, 1155–1166. doi: 10.1007/s00425-009-1014-3

Wei, J. Z., and Chatterton, N. J. (2001). Fructan biosynthesis and fructosyltransferase evolution: expression of the 6-SFT (sucrose: fructan 6-fructosyltransferase) gene in crested wheatgrass (*Agropyron cristatum*). *J. Plant Physiol.* 158, 1203–1213. doi: 10.1078/0176-1617-00241

Wiemken, A., Frehner, M., Keller, F., and Wagner, W. (1986). Fructan metabolism, enzymology and compartmentation. *Curr. Top. Plant Biochem. Physiol.* 5, 17–37. doi: 10.1007/s00018-009-0002-x

Wiemken, A., Sprenger, N., and Boller, T. (1995). "Fructan - an extension of sucrose by sucrose," in *Sucrose Metabolism, Biochemistry, Physiology, and Molecular Biology*, eds H. G. Pontis, G. L. Salerno, and E. S. Echeverria (American Society of Plant Physiologists, Rockville), 179–189. doi: 10.1007/BF00200301

Xue, G. P., Kooiker, M., Drenth, J., and McIntyre, C. L. (2011). TaMYB13 is a transcriptional activator of fructosyltransferase genes involved in β-2,6-linked fructan synthesis in wheat. *Plant J.* 68, 857–870. doi: 10.1111/j.1365-313X.2011.04737.x

Yoshida, M., Kawakami, A. and Van den Ende, W. (2007). "Graminan metabolism in cereals: wheat as a model system. in recent advances," in *Fructooligosaccharides*

Research, eds N. Shiomi, N. Benkeblia, and S. Onodera (Kerala, India: Research Signpost), 201–212. doi: 10.1104/pp.110.162222

Yuan, S., Le Roy, K., Venken, T., Lammens, W., Van den Ende, W., and De Maeyer, M. (2012). pKa modulation of the acid/base catalyst within GH32 and GH68: a role in substrate/inhibitor specificity? PLoS One 7:e37453. doi: 10.1371/journal.pone.0037453

Zhang, J., Dell, B., Conocono, E., Waters, I., Setter, T., and Appels, R. (2008). Water deficits in wheat: fructan exohydrolase (1-FEH) mRNA expression and relationship to soluble carbohydrate concentrations in two varieties. *New Phytol.* 181, 843–850. doi: 10.1111/j.1469-8137.2008.02713.x

Zimmermann, M. H., and Ziegler, H. (1975). "List of sugars and sugar alcohols in sieve-tube exudates," in *Transport in Plants 1: Phloem Transport. Encyclopedia of Plant Physiology, New Series,* Vol. 1, eds M. H. Zimmerman, J. A. Milburn (New York: Springer), 480–503. doi: 10.1104/pp.108.134791

Zuther, E., Kwart, M., Willmitzer, L., and Heyer, A. G. (2004). Expression of a yeast-derived invertase in companion cells results in long-distance transport of a trisaccharide in an apoplastic loader and influences sucrose transport. *Planta* 218, 759–766. doi: 10.1007/s00425-003-1148-7

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Post-translational regulation of sucrose transporters by direct protein–protein interactions

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Sucrose transporters are essential membrane proteins for the allocation of carbon resources in higher plants and protein–protein interactions play a crucial role in the post-translational regulation of sucrose transporters affecting affinity, transport capacity, oligomerization, localization, and trafficking. Systematic screening for protein interactors using sucrose transporters as bait proteins helped identifying several proteins binding to sucrose transporters from apple, *Arabidopsis*, potato, or tomato using the split ubiquitin system. This mini-review summarizes known sucrose transporter-interacting proteins and their potential function in plants. Not all of the identified interaction partners are postulated to be located at the plasma membrane, but some are predicted to be endoplasmic reticulum-residing proteins such as a protein disulfide isomerase and members of the cytochrome b5 family. Many of the SUT1-interacting proteins are secretory proteins or involved in metabolism. Identification of actin and actin-related proteins as SUT1-interacting proteins confirmed the observation that movement of SUT1-containing intracellular vesicles can be blocked by inhibition of actin polymerization using specific inhibitors. Manipulation of expression of these interacting proteins represents one possible way to modify resource allocation by post-translational regulation of sucrose transporters.

Keywords: protein–protein interaction, sucrose allocation, membrane microdomains, detergent-resistant membrane fraction, subcellular trafficking

INTRODUCTION

Elucidation of the interactome of membrane proteins seems to be a powerful tool to gain insights into signal transduction and regulation of nutrient transport in higher plants. For *Arabidopsis*, a robotic screening method was established based on the split ubiquitin system in yeast (Stagljar et al., 1998) in order to systematically analyze the membrane-based interactome with potential application in fungi, plants, and metazoan (Lalonde et al., 2010). This review will rather focus on single protein–protein interactions (PPIs) of plant sucrose transporters and summarize recent research in this field published during the past 5 years.

CO-LOCALIZED SUCROSE TRANSPORTERS IN THE PHLOEM CAN INTERACT WITH EACH OTHER

Sucrose transporters from potato and tomato belonging to different phylogenetic clades (Kühn and Grof, 2010) co-localize in phloem sieve elements (SEs; Barker et al., 2000; Weise et al., 2000; Reinders et al., 2002a). Using the split ubiquitin system it was shown that SlSUT1, SlSUT2, and SlSUT4 from tomato (*Solanum lycopersicum*) are able to form homo- and heterooligomers (Reinders et al., 2002a) and, even when expressed separately from different plasmids, the two halves of SlSUT1 were able to reconstitute a functional transporter (Reinders et al., 2002b).

Controversial localization of *Arabidopsis* sucrose transporters has been reported. Whereas AtSUC2 is assumed to be localized in phloem companion cells (CCs; Stadler and Sauer, 1996), AtSUC3/SUT2 was finally localized in phloem SEs (Meyer et al., 2004), and AtSUT4 was also detected in mesophyll protoplasts (Endler et al., 2006). Nevertheless, the promoter activity of the genes encoding all three sucrose transporters was mainly detected in phloem CCs (Schulze et al., 2003). This is not unexpected, since SE-specific sucrose transporter (SUT) expression is efficiently inhibited by help of a companion-cell-specific antisense construct (Kühn et al., 1996). *Arabidopsis* SUTs interact with each other in yeast cells (Schulze et al., 2003). Detection of strong promoter activity of AtSUT4 in phloem minor veins (Weise et al., 2000; Schulze et al., 2003) and the detection of AtSUT4 transcripts in mesophyll cells (Endler et al., 2006) argue for the presence of AtSUT4 in both tissues.

It should be also noted that the subcellular localization of SUT/SUCs does not completely overlap with each other. It is obvious that the subcellular localization of sucrose transporters is crucial for their functionality. Whereas StSUT1 localization is primarily at the plasma membrane (PM), SlSUT2 is also detectable in intracellular structures (**Figure 1**). SlSUT2 and StSUT1 interact completely different populations of proteins (Krügel and Kühn, unpublished results) suggesting different functions. The fact that SlSUT2–YFP fusion mainly localizes to intracellular membranes in

Abbreviations: BIFC, bimolecular fluorescence complementation; CC, companion cell; DRM, detergent-resistant membranes; MS, mass spectrometry; PDI, protein disulfide isomerase; PM, plasma membrane; PPI, protein–protein interaction; SE, sieve element; SUC, sucrose carrier; SUS, split ubiquitin system; SUT, sucrose transporter.

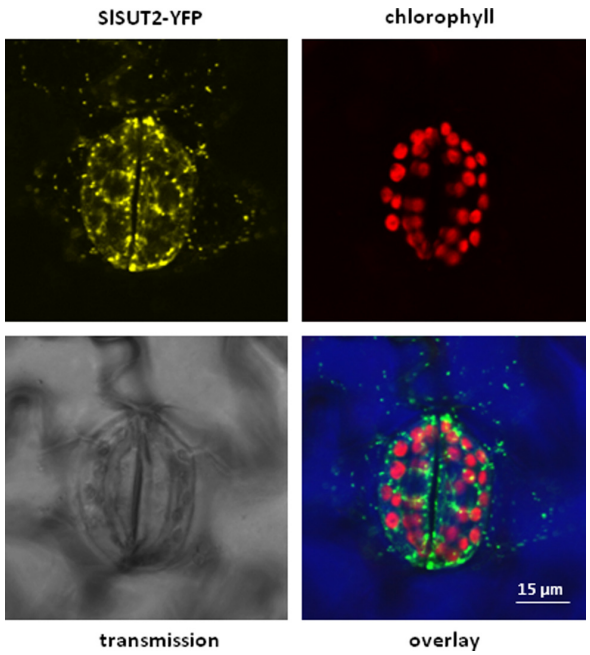

FIGURE 1 | Overlay picture showing subcellular distribution of a SlSUT2-YFP construct expressed under control of the constitutive CaMV 35S promoter in the vector pK7YWG2.0 (Karimi et al., 2002) in stably transformed tobacco plants (*Nicotiana tabacum*). SlSUT2-YFP fluorescence is mainly retained in intracellular structures, a possible argument for its non-functionality at the plasma membrane. Contrast of the overlay picture was enhanced by choosing the RGB mode leading to color modification. YFP fluorescence is therefore shown in green, chlorophyll autofluorescence is shown in red. Color of the transmission image is shown in blue.

stably transformed tobacco plants (**Figure 1**) might be one reason for its non-functionality as sucrose transporter in heterologous expression systems. It should be taken into consideration also for members of the SUT4 family that their subcellular localization is not necessarily static and confined to one single compartment (Chincinska et al., 2013), but undergoes dynamic changes during development, degradation or initial targeting to two different compartments.

The ability of StSUT1 to form dimers was confirmed experimentally by biochemical methods such as blue native PAGE, chemical cross-linking, two-dimensional gel electrophoresis, and bimolecular fluorescence complementation (BiFC; Krügel et al., 2008). Heterologous expression of StSUT1 from *Solanum tuberosum* revealed redox-dependent formation of homodimers (Krügel et al., 2008). The capacity of StSUT1 and StSUT4 to form heterodimers *in planta* was confirmed by BiFC where heterodimers were mainly detected in the endoplasmic reticulum (ER; Krügel et al., 2012). Thus heterodimer formation between SUTs can be found even when their subcellular localization does not completely overlap.

SUT1-INTERACTING PROTEINS

The yeast split ubiquitin system has been used to systematically screen for StSUT1-interacting proteins and seven different candidate proteins have been identified: a PDI, an enolase, an aldehyde dehydrogenase, a proton pyrophosphatase, an aquaporin, Snakin-1 (SN1), and an unknown protein (Krügel et al., 2012).

The attempts to confirm these interactions by co-immunoprecipitation (Co-IP) using specific antibodies or commercially available monoclonal antibodies raised against tagged and over-expressed SoSUT1 protein were not successful (Krügel et al., 2012), but large overlap was observed between proteins co-precipitated with StSUT1 and the detergent-resistant membrane (DRM) fraction of potato source leaf plasma membranes.

Immunoprecipitation was performed with either PMs from potato wild-type plants using an affinity-purified peptide antibody or alternatively with PMs from potato plants over-expressing a c-myc-tagged version of the sucrose transporter SoSUT1 from spinach (Leggewie et al., 2003) using monoclonal antibody against c-myc tag. Two candidates interact with StSUT1 from potato as well as with the over-expressed SoSUT1 from *Spinacia oleracea*: a plasma membrane H^+-ATPase (CAA54045) and a temperature-induced lipocalin (TIL; ABB02386). TILs are discussed to be involved in freezing tolerance of plants and/or in membrane stability and abiotic stress responses (Charron et al., 2005; Chi et al., 2009).

PROTEINS INVOLVED IN SUBCELLULAR PROTEIN TARGETING

StSUT1 was shown to undergo endocytosis in response to brefeldin A treatment and to be present in intracellular vesicles recycled at the PM. Motility of these vesicles is efficiently abolished by application of actin polymerization inhibitors such as latrunculin and actinomycin D (Liesche et al., 2010). This strongly suggests that movement of StSUT1-containing vesicles occurs in an actin-dependent manner involving the cellular cytoskeleton. Direct interaction of StSUT1 with actin and actin-related proteins strongly supports this hypothesis. The actin-related protein ARPC1/ARBC1A which was co-precipitated with StSUT1 (Krügel et al., 2012) is a component of the Arp2/3 complex implicated in the control of actin polymerization (Harries et al., 2005).

Also other StSUT1-interacting proteins identified by Co-IP are obviously involved in subcellular protein targeting and endosomal recycling at the PM. The integral membrane protein of the Yip1 family was shown to interact with the spinach SoSUT1 and is assumed to play a role in membrane trafficking. Yip1 family proteins are required for the biogenesis of ER-derived COPII vesicles in yeast and mammalian cells (Heidtman et al., 2005; Lorente-Rodriguez et al., 2009).

Other StSUT1-interacting proteins identified by Co-IP make part of the secretory pathway: Sec61 (NP_177993) is an ER-localized heterotrimeric protein translocator involved in the transport of proteins into and out of the ER (Rapoport, 1992) and mediates the export of misfolded proteins to the cytoplasm for degradation in various organisms (Pilon et al., 1997; Schmitz et al., 2000). Sec34 (NP_177485) is well known to regulate vesicular trafficking from ER to Golgi (Loh and Hong, 2002) and might be responsible for correct secretion of SUT1 to the plasma membrane.

SUT1-INTERACTING PROTEINS INVOLVED IN SIGNALING

Many other StSUT1-interacting proteins that have been identified either by SUS or by Co-IP are metabolic enzymes or involved in signaling or both.

The yeast Gal83 protein (CAB52141) is an important player under glucose limitation conferring specificity of the Snf1 complex to target proteins such as transcription factors (Yang et al., 1994). A correlation between Gal83 expression and assimilate transport in plants was described (Schwachtje et al., 2006).

A similar regulation of StSUT1 via phosphorylation as described for monosaccharide transporters in yeast can therefore be assumed (Carlson, 1998). Experimental data support the hypothesis of regulation of sucrose transporter activity via phosphorylation (Roblin et al., 1998; Nühse et al., 2004; Niittyla et al., 2007). 14-3-3 proteins are described to regulate many cellular processes by binding to phosphorylated proteins. The interaction of *Arabidopsis* 14-3-3 isoforms in response to changes in the plant nutrient status helped to identify new targets involved in nitrogen and sulfur metabolism (Shin et al., 2011). Among other sugar and mannitol transporters, AtSUC6 from *Arabidopsis* directly interacts with a 14-3-3χ protein of *Arabidopsis* suggesting that AtSUC6 is phosphorylated as well (Shin et al., 2011).

The detailed function of SUT phosphorylation remains to be elucidated. It is still unclear whether or not phosphorylation of the StSUT1 protein affects its subcellular localization or dimerization behavior.

METABOLISM

Adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase) is the key enzyme in starch biosynthesis and forms an allosteric heterotetramer. The activity of AGPase is regulated, e.g., by sugars and light through post-translational redox-dependent dimerization of the small subunit (Hendriks et al., 2003; Geigenberger et al., 2005).

Furthermore it is known from transgenic plants with reduced expression of plant SUTs that starch levels in source leaves are dramatically increased, which can be visualized macroscopically (Bürkle et al., 1998; Hackel et al., 2006) or by electron microscopy (Bürkle et al., 1998; Schulz et al., 1998; Chincinska et al., 2008). The identification of StSUT1-interacting proteins by Co-IP revealed direct interaction of StSUT1 with glucose-1-phosphate adenyltransferase (CAA53741) which represents the large subunit of the AGPase tetramer (Krügel et al., 2012). Therefore adaptation of starch biosynthetic capacity is not only possible via sugar availability and the redox-dependent dimerization of the small AGPase subunit but also by direct PPI between StSUT1 and the large subunit of AGPase.

REDOX-REGULATION: SNAKIN-1, PDI

One of the interaction partners of StSUT1 repeatedly identified in several independent screens was SN1 (Krügel et al., 2012). SN1 is a small cysteine-rich cell wall protein from *Solanum tuberosum* with an assumed antimicrobial function. It belongs to the Snakin/GASA (gibberellic acid stimulated in *Arabidopsis*) protein family and its physiological function was recently elucidated by generation of transgenic potato plants (Nahirnak et al., 2012a,b). SN1 is a PM protein and silencing of SN1 expression in transgenic potato plants led to reduced plant height and leaf size, whereas the mean cell size was increased. SN1 silencing affects cell division, cell wall composition, and leaf primary metabolism, i.e., the level of intermediates of the tricarboxylic acid (TCA) cycle. Redox homeostasis of SN1-silenced plants seems to be disturbed because the level of reactive oxygen species (ROS) is increased in these plants, while the level of ascorbate is reduced (Nahirnak et al., 2012b).

Computational prediction suggests that conserved oxidized cysteines in Snakin/GASA proteins can create up to five disulfide bridges that may act catalytically, i.e., playing a role in redox regulation exhibiting antioxidant activity (Wigoda et al., 2006). In *Arabidopsis*, the GASA4 protein affects the level of H_2O_2 and NO upon wounding and participates in GA-dependent signaling of flowering induction (Rubinovich and Weiss, 2010).

It is assumed that OsGSR1, a Snakin/GASA protein from rice, plays an important role in the cross-talk of GA and brassinosteroids (BR) signaling pathways (Wang et al., 2009). The expression of OsGSR1 is induced by gibberellins (Gas) and repressed by BR. Thus, members of the SN1/GASA protein family cover aspects in plant development, beyond their assumed function in response to biotic or abiotic stresses (Nahirnak et al., 2012b).

The physiological role of StSUT1–SN1 interaction in potato remains to be elucidated. A similar phenotype as in SN1-inhibited potato is observed when the expression of another SUT-interacting protein, the protein disulfide isomerase, is inhibited in transgenic potato plants by RNA interference (RNAi) silencing (E. Eggert and C. Kühn, unpublished results). In view of the fact that StSUT1 forms dimers in a redox-dependent manner (Krügel et al., 2008) it is interesting to note that two out of seven StSUT1-interacting proteins identified by SUS seem to be involved in redox homeostasis.

INTERACTORS OF SUT4 MEMBERS: MdSUT1 AND AtSUT4 INTERACT WITH PROTEINS OF THE cyb5 FAMILY

The MdSUT1 protein of *Malus domestica*, belonging to the SUT4 subfamily, was immunolocalized to the plasma membrane of apple phloem and parenchyma cells. A systematic SUS screen was performed using MdSUT1 and the sorbitol transporter MdSOT6 as bait proteins (Fan et al., 2009). Cytochrome b5 (Cyb5) was identified several times independently as interaction partners of MdSUT1 and of MdSOT6 (**Table 1**).

Cyb5s are small membrane-anchored proteins with a putative heme/steroid binding domain. They are involved in a number of oxidative reactions. Interaction between MdSUT1 and Cyb5 was confirmed by immunoprecipitation and BiFC. Since MdSUT1 was localized to the PM whereas Cyb5 is expected in the ER, electron microscopic immunolocalization was performed showing accurately that Cyb5 is localized in ER cisternae, which are connected to the PM (Fan et al., 2009).

β-Galactosidase (lacZ) activity tests in yeast have been used to quantify the strength of interaction in the presence or absence of glucose or sucrose in the medium. Increasing sucrose concentrations inhibited MdSUT1 interaction with Cyb5, whereas varying concentrations of glucose (which is not transported by MdSUT1) did not affect the strength of interaction (Fan et al., 2009).

Deletion constructs of the SUT-interacting Cyb5 revealed that the C-terminal region containing the membrane spanning domain of Cyb5 is essential for the interaction with sugar transporters,

Table 1 | Protein–protein interaction partners of plant sugar transporters identified by the yeast two hybrid split ubiquitin system.

Bait protein	Localization	Prey proteins	Confirmation	Reference
StSUT1 (*Solanum tuberosum*, SUT1 clade)	PM	Protein disulfide isomerase (PDI)	GST pull-down, BiFC, DRM protein, FRET acceptor bleaching, also interacts with SlSUT2 and StSUT4	Krügel et al. (2012)
		Snakin-1	Several times (>5) in independent screens	
		Inorganic pyro-phosphatase (PPi)	DRM protein	
		Tonoplast intrinsic protein (TIP)	DRM protein	
		Enolase	not confirmed	
		Aldehyde de-hydrogenase (ADH)	Several times in independent screens	
		Unknown protein	not confirmed	
MdSUT1 (*Malus domestica*, SUT4 clade)	PM	Cytochrome b5 (Cyb5)	BiFC, Co-IP, identified in 17 independent colonies	Fan et al. (2009)
MdSOT6 (*Malus domestica*, sorbitol transporter)	PM	Cyb5	BiFC, Co-IP, identified in 20 independent colonies	Fan et al. (2009)
AtSUT4 (*Arabidopsis thaliana*, SUT4 clade)	vacuole	Cyb5-1	BiCF, Co-IP	Li et al. (2012)
		Cyb5-2		
		Cyb5-3		
		Cyb5-4		
		Cyb5-6		

whereas the N-terminus only plays a role in the strength of the interaction. Thus, it is likely that the C-terminal part of Cyb5 interacts with the N-terminal part of MdSUT1 and that interaction takes place within the membrane. Co-expression of the Cyb5 protein with MdSUT1 in yeast increased affinity of MdSUT1 toward the substrate sucrose. Besides inhibiting PPI substitution of leucine$_{73}$ with proline abolished this increase in affinity. It was suggested that the sugar transporter–Cyb5 complex, which is strengthened under sugar starvation, promotes sugar uptake when sugar availability is limited.

In *Arabidopsis*, the same authors showed that AtSUT4, but not homologous transporters in the SUT1 or SUT2 subfamily, is able to interact with five different members of the Cyb5 family. Confirmation of interaction was obtained from pull-down assays and via BiFC (Li et al., 2012). Both *atsut4* and *cyb5-2* mutant plants show decreased sensitivity toward sucrose and glucose with respect to seed germination and it is suggested that the AtSUT4/Cyb5-2 complex might be involved in sensing or signaling not only of sucrose but also of glucose (Li et al., 2012).

SUMMARY AND OUTLOOK

PITFALLS

The identification of StSUT1-interacting proteins by different methods revealed completely different populations of interacting proteins. This is possibly explained by the differences of the two methods that have been used: for split ubiquitin screens, often a complete cDNA library is screened. In case of the StSUT1 split ubiquitin screen, the potato cDNA library was generated from RNA isolated and pooled from various tissues and developmental stages (Krügel et al., 2012). Thus, PPIs were demonstrated which under native conditions are not possible due to expression in different tissues, cells, developmental stages, and/or cellular compartments. PPIs determined using this method always need to be confirmed *in planta* in order to eliminate false-positive interaction partners.

Immunoprecipitation experiments, on the other hand, have been performed with plant extracts containing PM-enriched fractions isolated at a given developmental stage from source or sink leaves (Krügel et al., 2012). These interacting proteins are at least present at the same developmental stage and in the same isolated membrane fraction. Thus, a different population of proteins is expected for this screen of PPIs. Both sets of candidate genes identified with the two different methods need further confirmation.

THE POTENTIAL ROLE OF SUBCELLULAR COMPARTMENTATION

Elucidation of the StSUT1 interactome helped to get information about the regulation and subcellular localization of this SUT. Its presence in the DRM fraction was confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Krügel et al., 2012) and its association to lipid raft-like microdomains was visualized by expression of green fluorescent protein (GFP) fusion constructs in yeast (Krügel et al., 2008). SUT1 is constantly recycled at the SE PM in an actin-dependent manner (Liesche et al., 2010). Identification of actin-related proteins and components of the secretory pathway such as Sec34 and Sec61 among the StSUT1-interacting partners support these findings. A large overlap was observed between StSUT1-interacting proteins identified by Co-IP and proteins showing reduced detergent solubility (Krügel et al., 2012).

The question is now whether association of the StSUT1 to membrane microdomains is a prerequisite for endocytosis and recycling. Methyl-β-cyclodextrin (MβCD) causes sterol depletion

of the plant PM and thereby inhibits raft formation (Roche et al., 2008). The significantly increased activity of the glucose transporter GLUT1 in the presence of MβCD is explained by inhibition of endocytosis thereby increasing the number of transporters at the PM (Barnes et al., 2004; Caliceti et al., 2012). It is indispensable to test the impact of MβCD on the activity of SUT1, whose endocytosis seems to be inhibited by MβCD similarly to GLUT1 in the mammalian system (Liesche et al., 2010).

Raft association of plant membrane proteins may also impact lateral segregation. The potassium channel AtKAT1 was localized to microdomains and a low lateral mobility was observed (Reuff et al., 2010). Cholesterol depletion of the membrane decreases lateral mobility of PM proteins (Kwik et al., 2003) suggesting that association to raft-like microdomains enhance lateral diffusion.

Interestingly, the plasma membrane lining plasmodesmata share characteristics with membrane rafts, since typical raft proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins, the callose binding protein, remorin and also sphingolipids and phytosterols are enriched in the PM of plasmodesmata (Mongrand et al., 2010).

Translation of StSUT mRNAs is assumed to take place in CCs and the proteins need to be targeted to the PM of neighboring SEs (Kühn and Grof, 2010). Increased lateral mobility due to raft-like properties of the PM lining plasmodesmata might represent one possible way to facilitate correct targeting of SUTs from CC into enucleate SEs.

A recent paper, however, investigated lateral mobility of minimal membrane proteins by fluorescence recovery after photobleaching (FRAP) and points to the important role of the cell wall in immobilizing PM proteins, whereas association to membrane microdomains is assumed to play only a minor role in lateral mobility (Martiniere et al., 2012).

SIGNALING

Raft-like membrane compartments are regarded as organizing principle allowing attribution to protein-based sub-organellar compartments within a single cell to facilitate enzymatic reactions or cellular dynamics (Holthuis and Ungermann, 2012). Moreover, lipid rafts form cholesterol-enriched redox signaling membrane platforms providing an important driving force, e.g., for the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits to form (or activate) a functional enzyme complex in the PM (Jin et al., 2011). Depending on the cell type, sterol depletion of the PM using MβCD either delays NADPH oxidase activation leading to decreased ROS production (Vilhardt and van Deurs, 2004), decreased NADPH oxidase activity (Rao Malla et al., 2010), or promotes activation of NADPH oxidase (Han et al., 2008).

Lipid rafts are assumed to represent signaling platforms perhaps enabling membrane proteins to get in close contact to signaling proteins, kinases, phytohormone receptors, etc. However, the question remains whether the redox-dependent dimerization of SUT proteins and phosphorylation/dephosphorylation events are facilitated if the protein is concentrated in raft-like microdomains.

Manipulation of expression of SUT-interacting proteins represents a powerful tool to impact sucrose transport activity at the post-translational level in future experiments thereby strongly affecting carbon partitioning and resource allocation within crop plants.

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REFERENCES

Barker, L., Kühn, C., Weise, A., Schulz, A., Gebhardt, C., Hirner, B., et al. (2000). SUT2, a putative sucrose sensor in sieve elements. *Plant Cell* 12, 1153–1164.

Barnes, K., Ingram, J. C., Bennett, M. D., Stewart, G. W., and Baldwin, S. A. (2004). Methyl-beta-cyclodextrin stimulates glucose uptake in Clone 9 cells: a possible role for lipid rafts. *Biochem. J.* 378, 343–351. doi: 10.1042/BJ20031186

Bürkle, L., Hibberd, J. M., Quick, W. P., Kühn, C., Hirner, B., and Frommer, W. B. (1998). The H+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. *Plant Physiol.* 118, 59–68.

Caliceti, C., Zambonin, L., Prata, C., Vieceli Dalla Sega, F., Hakim, G., Hrelia, S., et al. (2012). Effect of plasma membrane cholesterol depletion on glucose transport regulation in leukemia cells. *PLoS ONE* 7:e41246. doi: 10.1371/journal.pone.0041246

Carlson, M. (1998). Regulation of glucose utilization in yeast. *Curr. Opin. Genet. Dev.* 8, 560–564. doi: 10.1016/S0959-437X(98)80011-7

Charron, J. B., Ouellet, F., Pelletier, M., Danyluk, J., Chauve, C., and Sarhan, F. (2005). Identification, expression, and evolutionary analyses of plant lipocalins. *Plant Physiol.* 139, 2017–2028. doi: 10.1104/pp.105.070466

Chi, W. T., Fung, R. W., Liu, H. C., Hsu, C. C., and Charng, Y. Y. (2009). Temperature-induced lipocalin is required for basal and acquired thermotolerance in *Arabidopsis*. *Plant Cell Environ.* 32, 917–927. doi: 10.1111/j.1365-3040.2009.01972.x

Chincinska, I., Gier, K., Krügel, U., Liesche, J., He, H., Grimm, B., et al. (2013). Photoperiodic regulation of the sucrose transporter StSUT4 affects the expression of circadian-regulated genes and ethylene production. *Front. Plant Sci.* 4:26. doi: 10.3389/fpls.2013.00026

Chincinska, I. A., Liesche, J., Krügel, U., Michalska, J., Geigenberger, P., Grimm, B., et al. (2008). Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response. *Plant Physiol.* 146, 515–528.

Endler, A., Meyer, S., Schelbert, S., Schneider, T., Weschke, W., Peters, S. W., et al. (2006). Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. *Plant Physiol.* 141, 196–207. doi: 10.1104/pp.106.079533

Fan, R. C., Peng, C. C., Xu, Y. H., Wang, X. F., Li, Y., Shang, Y., et al. (2009). Apple sucrose transporter SUT1 and sorbitol transporter SOT6 interact with cytochrome b5 to regulate their affinity for substrate sugars. *Plant Physiol.* 150, 1880–1901. doi: 10.1104/pp.109.141374

Geigenberger, P., Kolbe, A., and Tiessen, A. (2005). Redox regulation of carbon storage and partitioning in response to light and sugars. *J. Exp. Bot.* 56, 1469–1479. doi: 10.1093/jxb/eri178

Hackel, A., Schauer, N., Carrari, F., Fernie, A. R., Grimm, B., and Kühn, C. (2006). Sucrose transporter LeSUT1 and LeSUT2 inhibition affects tomato fruit development in different ways. *Plant J.* 45, 180–192.

Han, W., Li, H., Villar, V. A., Pascua, A. M., Dajani, M. I., Wang, X., et al. (2008). Lipid rafts keep NADPH oxidase in the inactive state in human renal proximal tubule cells. *Hypertension* 51, 481–487. doi: 10.1161/HYPERTENSIONAHA.107.103275

Harries, P. A., Pan, A., and Quatrano, R. S. (2005). Actin-related protein2/3 complex component ARPC1 is required for proper cell morphogenesis and polarized cell growth in *Physcomitrella patens*. *Plant Cell* 17, 2327–2339.doi: 10.1105/tpc.105.033266

Heidtman, M., Chen, C. Z., Collins, R. N., and Barlowe, C. (2005). Yos1p is a novel subunit of the

Yip1p–Yif1p complex and is required for transport between the endoplasmic reticulum and the Golgi complex. *Mol. Biol. Cell* 16, 1673–1683. doi: 10.1091/mbc.E04-10-0873

Hendriks, J. H., Kolbe, A., Gibon, Y., Stitt, M., and Geigenberger, P. (2003). ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of *Arabidopsis* and other plant species. *Plant Physiol.* 133, 838–849. doi: 10.1104/pp.103.024513

Holthuis, J. C., and Ungermann, C. (2012). Cellular microcompartments constitute general suborganellar functional units in cells. *Biol. Chem.* 394, 151–161. doi: 10.1515/hsz-2012-0265

Jin, S., Zhou, F., Katirai, F., and Li, P. L. (2011). Lipid raft redox signaling: molecular mechanisms in health and disease. *Antioxid. Redox Signal.* 15, 1043–1083. doi: 10.1089/ars.2010.3619

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7, 193–195.

Krügel, U., He, H. X., Gier, K., Reins, J., Chincinska, I., Grimm, B., et al. (2012). The potato sucrose transporter StSUT1 interacts with a DRM-associated protein disulfide isomerase. *Mol. Plant* 5, 43–62. doi: 10.1093/mp/ssr048

Krügel, U., Veenhoff, L. M., Langbein, J., Wiederhold, E., Liesche, J., Friedrich, T., et al. (2008). Transport and sorting of the *Solanum tuberosum* sucrose transporter SUT1 is affected by post-translational modification. *Plant Cell* 20, 2497–2513. doi: 10.1105/tpc.108.058271

Kühn, C., and Grof, C. P. (2010). Sucrose transporters of higher plants. *Curr. Opin. Plant Biol.* 13, 288–298. doi: 10.1016/j.pbi.2010.02.001

Kühn, C., Quick, W. P., Schulz, A., Riesmeier, J. R., Sonnewald, U., and Frommer, W. B. (1996). Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ.* 19, 115–1123. doi: 10.1111/j.1365-3040.1996.tb00426.x

Kwik, J., Boyle, S., Fooksman, D., Margolis, L., Sheetz, M. P., and Edidin, M. (2003). Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13964–13969. doi: 10.1073/pnas.2336102100

Lalonde, S., Sero, A., Pratelli, R., Pilot, G., Chen, J., Sardi, M. I., et al. (2010). A membrane protein/signaling protein interaction network for *Arabidopsis* version AMPv2. *Front. Physiol.* 1:24. doi: 10.3389/fphys.2010.00024

Leggewie, G., Kolbe, A., Lemoine, R., Roessner, U., Lytovchenko, A., Zuther, E., et al. (2003). Overexpression of the sucrose transporter SoSUT1 in potato results in alterations in leaf carbon partitioning and in tuber metabolism but has little impact on tuber morphology. *Planta* 217, 158–167.

Li, Y., Li, L. L., Fan, R. C., Peng, C. C., Sun, H. L., Zhu, S. Y., et al. (2012). *Arabidopsis* sucrose transporter SUT4 interacts with cytochrome b5-2 to regulate seed germination in response to sucrose and glucose. *Mol. Plant* 5, 1029–1041. doi: 10.1093/mp/sss001

Liesche, J., He, H. X., Grimm, B., Schulz, A., and Kühn, C. (2010). Recycling of Solanum sucrose transporters expressed in yeast, tobacco, and in mature phloem sieve elements. *Mol. Plant* 3, 1064–1074. doi: 10.1093/mp/ssq059

Loh, E., and Hong, W. (2002). Sec34 is implicated in traffic from the endoplasmic reticulum to the Golgi and exists in a complex with GTC-90 and ldlBp. *J. Biol. Chem.* 277, 21955–21961. doi: 10.1074/jbc.M202326200

Lorente-Rodriguez, A., Heidtman, M., and Barlowe, C. (2009). Multicopy suppressor analysis of thermosensitive YIP1 alleles implicates GOT1 in transport from the ER. *J. Cell Sci.* 122, 1540–1550. doi: 10.1242/jcs.042457

Martiniere, A., Lavagi, I., Nageswaran, G., Rolfe, D. J., Maneta-Peyret, L., Luu, D. T., et al. (2012). Cell wall constrains lateral diffusion of plant plasma-membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12805–12810. doi: 10.1073/pnas.1202040109

Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R. D., and Sauer, N. (2004). Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. *Plant Physiol.* 134, 684–693. doi: 10.1104/pp.103.033399

Mongrand, S., Stanislas, T., Bayer, E. M., Lherminier, J., and Simon-Plas, F. (2010). Membrane rafts in plant cells. *Trends Plant Sci.* 15, 656–663. doi: 10.1016/j.tplants.2010.09.003

Nahirnak, V., Almasia, N. I., Fernandez, P. V., Hopp, H. E., Estevez, J. M., Carrari, F., et al. (2012a). Potato snakin-1 gene silencing affects cell division, primary metabolism, and cell wall composition. *Plant Physiol.* 158, 252–263. doi: 10.1104/pp.111.186544

Nahirnak, V., Almasia, N. I., Hopp, H. E., and Vazquez-Rovere, C. (2012b). Snakin/GASA proteins: involvement in hormone crosstalk and redox homeostasis. *Plant Signal. Behav.* 7, 1004–1008. doi: 10.4161/psb.20813

Niittyla, T., Fuglsang, A. T., Palmgren, M. G., Frommer, W. B., and Schulze, W. X. (2007). Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of *Arabidopsis*. *Mol. Cell. Proteomics* 6, 1711–1726. doi: 10.1074/mcp.M700164-MCP200

Nühse, T. S., Stensballe, A., Jensen, O. N., and Peck, S. C. (2004). Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell* 16, 2394–2405. doi: 10.1105/tpc.104.023150

Pilon, M., Schekman, R., and Romisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* 16, 4540–4548. doi: 10.1093/emboj/16.15.4540

Rao Malla, R., Raghu, H., and Rao, J. S. (2010). Regulation of NADPH oxidase (Nox2) by lipid rafts in breast carcinoma cells. *Int. J. Oncol.* 37, 1483–1493.

Rapoport, T. A. (1992). Transport of proteins across the endoplasmic reticulum membrane. *Science* 258, 931–936.doi: 10.1126/science.1332192

Reinders, A., Schulze, W., Kuhn, C., Barker, L., Schulz, A., Ward, J. M., et al. (2002a). Protein–protein interactions between sucrose transporters of different affinities colocalized in the same enucleate sieve element. *Plant Cell* 14, 1567–1577. doi: 10.1105/tpc.002428

Reinders, A., Schulze, W., Thaminy, S., Stagljar, I., Frommer, W. B., and Ward, J. M. (2002b). Intra- and intermolecular interactions in sucrose transporters at the plasma membrane detected by the split-ubiquitin system and functional assays. *Structure* 10, 763–772. doi: 10.1016/S0969-2126(02)00773-6

Reuff, M., Mikosch, M., and Homann, U. (2010). Trafficking, lateral mobility and segregation of the plant K channel KAT1. *Plant Biol. (Stuttg.)* 12(Suppl. 1), 99–104. doi: 10.1111/j.1438-8677.2010.00355.x

Roblin, G., Sakr, S., Bonmort, J., and Delrot, S. (1998). Regulation of a plant plasma membrane sucrose transporter by phosphorylation. *FEBS Lett.* 424, 165–168. doi: 10.1016/S0014-5793(98)00165-3

Roche, Y., Gerbeau-Pissot, P., Buhot, B., Thomas, D., Bonneau, L., Gresti, J., et al. (2008). Depletion of phytosterols from the plant plasma membrane provides evidence for disruption of lipid rafts. *FASEB J.* 22, 3980–3991. doi: 10.1096/fj.08-111070

Rubinovich, L., and Weiss, D. (2010). The *Arabidopsis* cysteine-rich protein GASA4 promotes GA responses and exhibits redox activity in bacteria and in planta. *Plant J.* 64, 1018–1027. doi: 10.1111/j.1365-313X.2010.04390.x

Schmitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol.* 148, 1203–1212. doi: 10.1083/jcb.148.6.1203

Schulz, A., Kühn, C., Riesmeier, J. R., and Frommer, W. B. (1998). Ultrastructural effects in potato leaves due to antisense-inhibition of the sucrose transporter indicate an apoplasmic mode of phloem loading. *Planta* 206, 533–543.

Schulze, W. X., Reinders, A., Ward, J., Lalonde, S., and Frommer, W. B. (2003). Interactions between co-expressed *Arabidopsis* sucrose transporters in the split-ubiquitin system. *BMC Biochem.* 4:3. doi: 10.1186/1471-2091-4-3

Schwachtje, J., Minchin, P. E., Jahnke, S., van Dongen, J. T., Schittko, U., and Baldwin, I. T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12935–12940. doi: 10.1073/pnas.0602316103

Shin, R., Jez, J. M., Basra, A., Zhang, B., and Schachtman, D. P. (2011). 14-3-3 proteins fine-tune plant nutrient metabolism. *FEBS Lett.* 585, 143–147. doi: 10.1016/j.febslet.2010.11.025

Stadler, R., and Sauer, N. (1996). The *Arabidopsis thaliana* AtSUC2 gene is specifically expressed in companion cells. *Bot. Acta* 109, 299–306.

Stagljar, I., Korostensky, C., Johnsson, N., and te Heesen, S. (1998). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5187–5192. doi: 10.1073/pnas.95.9.5187

Vilhardt, F., and van Deurs, B. (2004). The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. *EMBO J.* 23, 739–748. doi: 10.1038/sj.emboj.7600066

Wang, L., Wang, Z., Xu, Y., Joo, S. H., Kim, S. K., Xue, Z., et al. (2009). OsGSR1 is involved in crosstalk between gibberellins and brassinosteroids in rice. *Plant J.* 57, 498–510. doi: 10.1111/j.1365-313X.2008.03707.x

Weise, A., Barker, L., Kühn, C., Lalonde, S., Buschmann, H., Frommer, W. B., et al. (2000). A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. *Plant Cell* 12, 1345–1355.

Wigoda, N., Ben-Nissan, G., Granot, D., Schwartz, A., and Weiss, D. (2006). The gibberellin-induced, cysteine-rich protein GIP2 from Petunia hybrida exhibits in planta antioxidant activity. *Plant J.* 48, 796–805. doi: 10.1111/j.1365-313X.2006.02917.x

Yang, X., Jiang, R., and Carlson, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. *EMBO J.* 13, 5878–5886.

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Role of metabolite transporters in source–sink carbon allocation

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Plants assimilate carbon dioxide during photosynthesis in chloroplasts. Assimilated carbon is subsequently allocated throughout the plant. Generally, two types of organs can be distinguished, mature green source leaves as net photoassimilate exporters, and net importers, the sinks, e.g., roots, flowers, small leaves, and storage organs like tubers. Within these organs, different tissue types developed according to their respective function, and cells of either tissue type are highly compartmentalized. Photoassimilates are allocated to distinct compartments of these tissues in all organs, requiring a set of metabolite transporters mediating this intercompartmental transfer. The general route of photoassimilates can be briefly described as follows. Upon fixation of carbon dioxide in chloroplasts of mesophyll cells, triose phosphates either enter the cytosol for mainly sucrose formation or remain in the stroma to form transiently stored starch which is degraded during the night and enters the cytosol as maltose or glucose to be further metabolized to sucrose. In both cases, sucrose enters the phloem for long distance transport or is transiently stored in the vacuole, or can be degraded to hexoses which also can be stored in the vacuole. In the majority of plant species, sucrose is actively loaded into the phloem via the apoplast. Following long distance transport, it is released into sink organs, where it enters cells as source of carbon and energy. In storage organs, sucrose can be stored, or carbon derived from sucrose can be stored as starch in plastids, or as oil in oil bodies, or – in combination with nitrogen – as protein in protein storage vacuoles and protein bodies. Here, we focus on transport proteins known for either of these steps, and discuss the implications for yield increase in plants upon genetic engineering of respective transporters.

Keywords: source–sink, carbon allocation, metabolite transporters, yield, storage

LIMITATIONS FOR PLANT YIELD AND GENERAL CONSIDERATIONS

Humans often exploit plant organs that serve plants for their persistence. Organs such as seeds, roots, tubers, or fruits contain considerable energy stored as oil, protein, sugar, or starch that allow the plant to start a new life cycle or its offspring to germinate. Seeds frequently are accompanied by or are embedded in fruits which also contribute to reproduction in that animals eat and often spread these fruits. Humans use these nutritious organs as food or feed and thousands of years ago had already started to propagate plants with better qualities by selective breeding. After long phases of selection-, classical and recently SMART (Selection with Markers and Advanced Reproductive Technologies; McCouch, 2004) breeding, the limits of quality or yield enhancement might be almost reached for some crops. In addition to these breeding programs, transgenic plants have been successfully engineered that often outcompete classically bred cultivars, e.g., plants with enhanced resistances against pathogens or herbicides. Although sometimes unacknowledged, especially by the European society, future prospects for transgenic plants being a farmer's first choice are intact. It can be assumed that new generations of transgenic plants also include those which are genetically engineered in their source- and/or sink capacity. These features are closely linked to the harvest index, i.e., weight of the grain (or other harvestable organs) divided by the total plant weight. Indeed, genetically engineered crop plants with changes in source- and/or sink capacities were created which were either impaired (e.g., Riesmeier et al., 1994) or improved in yield (e.g., Jonik et al., 2012), demonstrating the feasibility and putative impact of such approaches on yield increase.

In general, the yield of a given cultivar is limited by several factors, and yield can only be improved by unrestricting these limitations. The challenge therefore is to know the limiting factor(s) of the respective crop.

This view, however, should be extended by referring to what is known about the cross-talk between source- and sink activities. When carbon reserves of storage organs are used up by the newly emerging seedling, photosynthesis begins and atmospheric CO_2 is assimilated. Given an otherwise optimal supply with water, nitrogen, and other nutrients, only the rate of CO_2 fixation determines plant growth during the vegetative phase. During further development toward maturation, storage organs are produced. The total of all sink organs then additionally determines the photosynthetic rate, as decreasing demand of the sink tissues for carbon ultimately

limits photosynthesis, a phenomenon known as "sink limitation of photosynthesis" (Stitt, 1991; Paul and Foyer, 2001; Paul and Pellny, 2003; Ainsworth and Bush, 2011). Therefore, retention of the sink tissues to draw photosynthetic carbon would maintain photosynthesis (McCormick et al., 2006, 2008).

Here, we describe the allocation of carbon from source- to sink tissues and focus on the metabolite transporters known to be involved. We will also focus on C3 plants as the majority of these transporters have been described from these plant species.

ALLOCATION OF CARBON WITHIN SOURCE LEAVES

Production of carbon compounds occurs during photosynthesis in the chloroplasts. When the assimilated carbon exceeds the local need, carbon can then be exported. Net photoassimilate exporting leaves are defined as "source" (Turgeon, 1989). However, assimilated carbon can either be transiently stored as starch in chloroplasts or transported to the cytosol. From there, it can be directly exported from the leaf or transported into and transiently stored in the vacuole. From both compartments, chloroplasts and the vacuole, carbon can be exported when sink demand exceeds the actual production via photosynthesis, e.g., and of course during the diurnal cycle (starch release from the chloroplast at night). Transient storage of assimilated carbon in source leaves during the day could be regarded as a valve for excess carbon production and might be important for maintaining a high photosynthesis rate. At the same time, plants need this transiently stored carbon pool during the night to maintain sucrose formation for metabolic processes.

CHLOROPLAST TRANSPORTERS

During photosynthesis CO_2 is assimilated in a series of reactions known as the Calvin–Benson cycle. One of six triose phosphates produced can either be used for starch production or can be exported from chloroplasts to the cytosol for sucrose synthesis or respiration. This export is accomplished by the triose phosphate/phosphate translocator (TPT; "1" in **Figure 1**; Fliege et al., 1978; Flügge et al., 1989) which exports triose phosphates in counter-exchange with orthophosphate (day path of carbon). Knock-out and -down *tpt* mutants and transgenic plants produce high amounts of starch during the day which are then partially degraded again during the day (*Arabidopsis*, tobacco; Häusler et al., 1998, 2000; Schneider et al., 2002; Walters et al., 2004; Schmitz et al., 2012) or the following night period (potato; Riesmeier et al., 1993a; Heineke et al., 1994). By this, *tpt* mutants and antisense plants manage to grow normally without any aberrant phenotype. Overexpression of the TPT was performed in tobacco using the *Flaveria trinervia TPT* gene (Häusler et al., 2000) and in *Arabidopsis* using the endogenous gene (Cho et al., 2012) with only minor effects. When a cytosolic fructose 1,6-bisphosphatase (cFBPase) was simultaneously overexpressed, *Arabidopsis* plants were larger

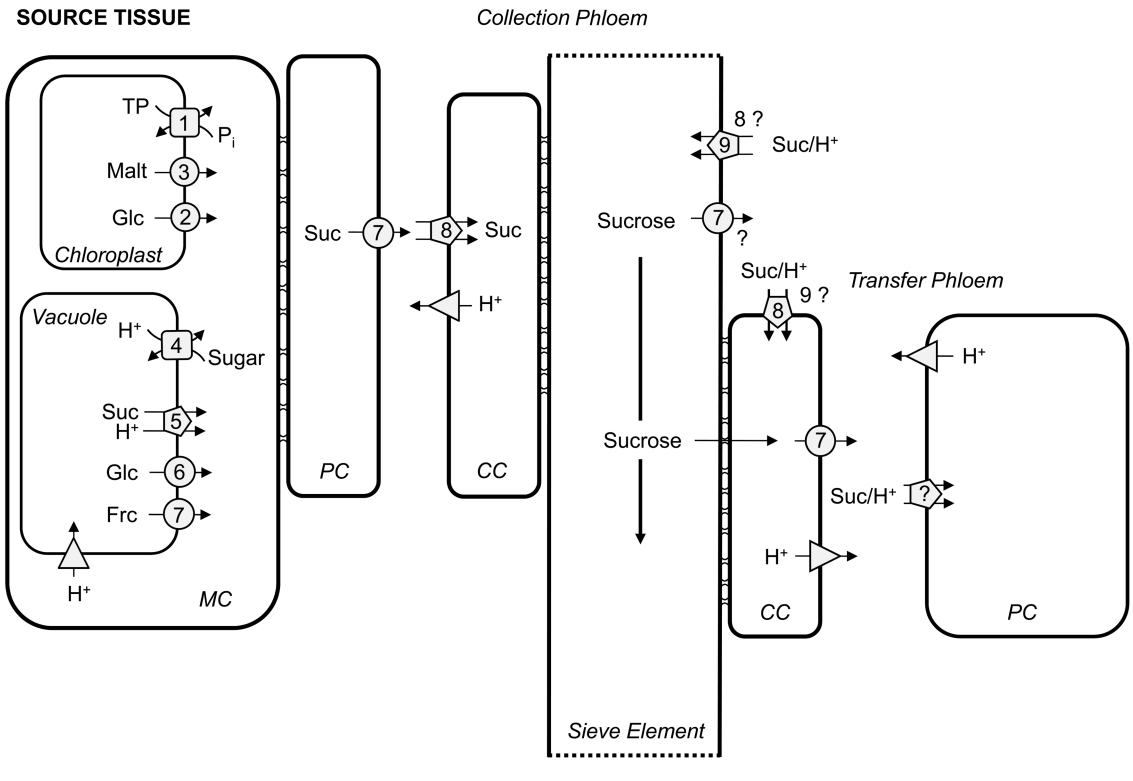

FIGURE 1 | Overview of carbon transport proteins in source leaves. Transport steps in mesophyll cells (MC), parenchyma cells (PC), companion cells (CC), and sieve elements are depicted indicating their respective mode of action. Squares represent antiporters, circles describe facilitators, pentagons depict symporters, and triangles H^+-ATPases/PPases. 1, TPT; 2, pGlcT; 3, MEX; 4, TMT and VGT; 5, SUC4/SUT4; 6, ESL1; 7, SWEETs; 8, SUC2/SUT1; 9, SUC3. TP, triose phosphates; Malt, maltose; Glc, glucose; Suc, sucrose; Frc, fructose. Please refer to text for transporter abbreviations.

and exhibited a higher photosynthetic capacity (Cho et al., 2012). Unfortunately, no information on seed yield was provided. Thus, from this approach it cannot be deduced whether *Arabidopsis* yield could possibly be source-limited.

During the night, carbon export from the chloroplast starts with the breakdown of transitory starch and then export of the resulting products maltose and glucose. Weise et al. (2004) found evidence for maltose as the main product exported from chloroplasts at night and proposed a maltose transporter in addition to the plastid glucose transporter (pGlcT; "2" in **Figure 1**; Weber et al., 2000). Export of maltose is mediated by the maltose transporter MEX1 ("3" in **Figure 1**; Niittylä et al., 2004). The loss-of-function "maltose excess" mutant *mex1* was impaired in growth, had a light green yellowish appearance and accumulated maltose and starch. Overexpression of the endogenous transporter (Niittylä et al., 2004) as well as the apple (*Malus domestica*) transporter (Reidel et al., 2008) complemented the mutant phenotype. None of the lines that rescued the *mex1* phenotype displayed any additional aberrant phenotype indicating that *MEX1* overexpression would not lead to increased growth or increased source capacity. Double *mex1*/*tpt-2* or *mex1*/*pglct* but not *pglct*/*tpt-2* mutants displayed an even more severe growth phenotype than *mex1* (Cho et al., 2011) as well as a combination of starch synthesis (*adg1-1*) or degradation (*starch-excess 1-3*, *sex1-3*) mutants with *tpt* mutants (Schneider et al., 2002). The severity of the respective phenotypes differed and the underlying reason is not yet fully understood, however, this might include retrograde signals from the chloroplasts to the nucleus (Stettler et al., 2009; Schmitz et al., 2012).

VACUOLAR TRANSPORTERS

In source leaves, sugar can be temporarily stored in vacuoles, e.g., when sucrose export via the phloem is saturated (Martinoia et al., 2000). The designation of sugar storage as "temporary" implies the existence of vacuolar sugar importers and exporters. The types of putative sugar importers known to date have been characterized from *Arabidopsis thaliana*, tonoplast monosaccharide transporters (TMTs; "4" in **Figure 1**; Wormit et al., 2006) and vacuolar glucose transporters (VGTs; "4" in **Figure 1**; Aluri and Büttner, 2007). Both types of transporters belong to the monosaccharide transporter (-like) gene family, form a sub-clade each (Büttner, 2007, 2010) and mediate an energy-dependent transport driven by V-type H^+-ATPases and vacuolar H^+-PPases. The transport direction by patch clamp technique has been shown to be sugar import into vacuoles for the TMTs (Wingenter et al., 2010; Schulz et al., 2011), while the function of VGT1 in seed germination and flowering as revealed by analyzing vgt1 mutant plants also indicated glucose import into vacuoles (Aluri and Büttner, 2007). TMTs have been shown to import sugar in a proton antiport manner (Wingenter et al., 2010; Schulz et al., 2011), and putative VGT1-mediated glucose import is likely to be also coupled to proton antiport (Aluri and Büttner, 2007). Whereas initially and in accordance with their name TMTs were thought to transport monosaccharides across the tonoplast membrane (Wormit et al., 2006; Wingenter et al., 2010) recently it has been shown that also sucrose is transported by TMTs (Schulz et al., 2011). The *Arabidopsis* genome contains three TMT genes indicating possible redundancy for TMT function. Indeed, when analyzing loss-of-function tmt mutants, only tmt1/tmt2/tmt3 triple or tmt1/tmt2 double knock-out mutants showed impaired uptake of glucose into isolated mesophyll vacuoles (Wormit et al., 2006) or reduced sugar-induced changes in currents in patch clamp analyses (Wingenter et al., 2010), respectively. Double mutants grew worse, compared to wild-type, and were also impaired in seed yield, whereas double mutants additionally overexpressing the *Arabidopsis* TMT1 under the control of the 35S-CaMV promoter "over"-rescued the mutant phenotype due to higher TMT activity. This led to larger plants with increased seed yield. It was hypothesized that sugar sensing is diminished in overexpressing lines due to an increased import of sugars into the vacuole and – more importantly – out of the cytosol. These findings might indicate *Arabidopsis* "yield" to be source-limited (Wingenter et al., 2010). A sink effect, however, cannot be ruled out when using a constitutive promoter for the overexpression of TMT1. One could also imagine a sink effect, in that, sugar is transiently stored in vacuoles of embryo cells to perpetuate sucrose flow to sinks and thus increase sink strength.

Sugar export from the vacuole, on the contrary, is likely to occur in a proton symport manner or, when subcellular concentrations of the sugar to be transported allow for it, can occur by facilitated diffusion. To avoid a futile cycling of sugars into and out of the vacuole and thereby diminishing the proton gradient across the tonoplast, at least one of these processes should be regulated. Indeed, there is evidence for post-translational regulation of TMTs by phosphorylation. A member of the mitogen-activated protein 3-kinase called VH1-interacting kinase (VIK) was found to activate TMTs (Wingenter et al., 2011).

Sucrose transporters of type IV (SUC4/SUT4; "5" in **Figure 1**; Sauer, 2007; Kühn and Grof, 2010; Payyavula et al., 2011) of several species have been found to localize to the vacuolar membrane (Endler et al., 2006; Reinders et al., 2008; Eom et al., 2011; Payyavula et al., 2011; Schneider et al., 2012). The same clade has been designated "type III" by Reinders et al. (2012). Some of these sucrose transporters have been reported to localize to the plasma membrane (Weise et al., 2000; Weschke et al., 2000; Chincinska et al., 2008; Kühn and Grof, 2010). Although still under debate, we only discuss the function of this group of sucrose transporters as vacuolar transporters here. Schulz et al. (2011) were able to measure sucrose-driven proton import into the vacuole under an inverted pH gradient when overexpressing a functional AtSUC4–GFP (green fluorescent protein) construct in the background of the quadruple *tmt1*/*tmt2*/*vgt1*/*vgt2* mutant – to overcome sugar/H^+-antiport activities – using the patch clamp technique. This result can only be interpreted as sucrose/H^+ co-export from vacuoles mediated by SUC4 *in vivo*. An *Arabidopsis suc4* loss-of-function mutant did not display any aberrant phenotype whereas constitutive SUC4 overexpressed seedlings had slightly less sucrose than wild-type (Schneider et al., 2012). The possible impact of SUC4 under- or overexpressing plants on seed yield was not reported by the authors. In contrast, *sut2* mutants of rice (OsSUT2 belonging to clade IV) had a reduced sugar export ability and accumulated sugars in source leaves which led to several aberrant phenotypes, among them growth retardation, reduction in tiller number and decreased grain weight (Eom et al., 2011). These findings are in line with the interpretation that SUT2-mediated export of sucrose from vacuoles is important to sustain

source capacity in rice. It remains to be elucidated whether or not the overexpression of SUT2 in rice leads to increased grain yield.

Evidence for a tonoplast hexose exporter has been gathered by Yamada et al. (2010). The authors mistargeted a slightly mutagenized *Arabidopsis* ESL1, a tonoplast-residing member of the ERD6-like monosaccharide transporters ("6" in **Figure 1**; Büttner, 2007, 2010), to the plasma membrane of tobacco BY-2 cells. Glucose transport across the membrane could be measured using ^{14}C-glucose. Addition of high concentrations of unlabeled hexoses inhibited ^{14}C-glucose transport to different extents depending on the sugar used for inhibition. The transport was found to be independent of a proton gradient, and determination of the K_m for glucose revealed ESL to be a low affinity facilitator exporting monosaccharides out of the vacuole (Yamada et al., 2010). *ESL1* is expressed upon drought and salt stress, as are vacuolar invertases that produce hexoses from sucrose. ESL1-mediated export of hexoses from vacuoles might contribute to the plant's attempt to increase the osmotic potential within cells. The *esl1* mutant, however, did not display any aberrant phenotype, perhaps due to redundancy (Yamada et al., 2010).

Another *Arabidopsis* ERD6-like monosaccharide transporter, ERDL6, has been described as vacuolar glucose exporter by Poschet et al. (2011). Expression of *ERDL6* is induced when sugars are mobilized, e.g., in darkness, upon heat stress or wounding, and is reduced upon cold stress or external feeding of sugars to the plant. Elevated levels of glucose, but not of fructose and sucrose, were found in *erdl6* mutants, and non-aqueous fractionation revealed a more pronounced glucose increase in vacuoles compared to extra-vacuolar compartments. The opposite effect of lower glucose contents, however, was described for overexpressors either of the endogenous or a homologous sugar beet transporter (BvIMP). Knock-out *erdl6* mutants, however, displayed increased seed yield with elevated sugar, protein, and lipid contents of mature seeds, as had been described for TMT1 overexpressors (Wingenter et al., 2010). It remains elusive if yield increase can be attributed to source or sink effects since *ERDL6* is expressed in leaves and seeds, according to the eFP browser (Winter et al., 2007). It also remains to be elucidated whether the mechanism of ERDL6-mediated transport is facilitation – as found for ESL1 – or is secondary transport.

Only very recently, the *Arabidopsis* vacuolar sugar exporter SWEET17 has been discovered with help of a quantitative genetics approach ("7" in **Figure 1**; Chardon et al., 2013). SWEET17 is the first protein among the SWEET transporter family localized to the tonoplast, all other SWEETs described previously were localized in the plasma membrane (Chen et al., 2010, 2012). Moreover, SWEET17 is the first fructose transporter, other SWEET family members facilitate diffusion of glucose or sucrose (Chen et al., 2010, 2012). The authors were able to demonstrate uptake or efflux of ^{13}C-labeled fructose upon expression of SWEET17 in *Xenopus* oocytes. In *sweet17* mutants, fructose contents were enhanced compared to wild-type, and accumulation of fructose was more pronounced in nitrogen-limited or cold-stressed plants. Both growth conditions led to an elevated uptake of sugars into vacuoles mediated by TMTs (Wingenter et al., 2010; Schulz et al., 2011), and fructose appears to not be released from *sweet17* mutant vacuoles. SWEET17 expression was found in leaves, with the strongest level in xylem vascular tissue, pointing toward a function in fructose allocation within the plant. Accordingly, the only subtle phenotype of *sweet17* mutants was in the reduced size of flower stalks and slightly reduced seed yield (Chardon et al., 2013).

PHLOEM LOADING, LEAKAGE, AND RETRIEVAL

When sucrose is exported from source leaves, it has to enter the phloem. Several strategies for the loading of sucrose (and other sugars and sugar alcohols) have been described (for a review, see Rennie and Turgeon, 2009). We have restricted ourselves here to apoplastic sucrose loading when describing the route sucrose takes from source leaves to sinks.

SWEET-FACILITATED SUCROSE EFFLUX INTO THE APOPLAST

For many years it remained unknown how sucrose is released from mesophyll cells or phloem parenchyma cells into the apoplast prior to loading into the companion cell/sieve element complex. It was recently demonstrated that this release is facilitated by members of the SWEET transporter family, i.e., SWEET11 and -12 from *Arabidopsis* and SWEET11 and -14 from rice ("7" in **Figure 1**; Chen et al., 2012). Facilitation of sucrose efflux without energy consumption can occur due to the concentration gradient between the mesophyll cell/phloem parenchyma cell symplastic continuum and the apoplast has been described to be steep [40-, 150-, and 750-fold in spinach, barley (Lohaus et al., 1995), and sugar beet (Fondy and Geiger, 1977; Lohaus et al., 1994), respectively]. Closing of this gap in source sink carbon allocation was of great importance and accordingly was effusively acclaimed (Baker et al., 2012; Braun, 2012) not only because of the completion of a pathway but also due to enabling new perspectives with respect to genetic engineering of the SWEET proteins. Before sucrose transporting SWEETs had been discovered, other members of the family were found to facilitate efflux of glucose into the apoplast fulfilling a potential role in feeding pathogens residing in the cell wall (Chen et al., 2010) with maybe even higher potential for the agricultural industry (Sonnewald, 2011; Baker et al., 2012).

Whereas, single knock-out mutants of AtSWEET11 and -12 did not display any obvious altered phenotype, *Arabidopsis sweet11*/*sweet12* double mutants did display an expected phenotype of blocked phloem loading, i.e., smaller plants, elevated levels of leaf starch and sucrose, a reduced export of fixed ^{14}C from leaves and reduced growth of sinks (e.g., roots; Chen et al., 2012). Rice *SWEET11* and *-14* were transcriptionally induced upon infection with *Xanthomonas oryzae* pv. *oryzae*. This induction is mediated through pathogen-borne effectors binding to *SWEET11* and *-14* promoters. Mutations in the binding sites led to resistance against the pathogen (Antony et al., 2010). This finding indicated that increasing SWEET activity would lead to more sucrose in the apoplast on which pathogens could feed and spread. Thus, an overexpression of SWEETs in non-infected plants would most likely also lead to an increase in apoplastic sucrose content. Whether or not SWEET overexpression could enhance source capacity would depend on the plasticity of SUC2/SUT1 transporter ("8" in **Figure 1**) activity importing sucrose from the apoplast into phloem companion cells in a sufficient capacity.

PHLOEM LOADING OF SUCROSE

Sucrose is loaded into phloem companion cells and is transported in sieve elements along a hydrostatic pressure gradient driven by active sucrose loading. This enables a mass flow to sink organs with higher pressure in the source leaves and lower pressure in the sinks where sucrose is then unloaded from the phloem (Münch, 1930).

Because of the extreme difference in sucrose concentration between apoplast and phloem, sucrose loading against this gradient [630-, 700-, and 15,000-fold in spinach, barley (Lohaus et al., 1995), and sugar beet (Fondy and Geiger, 1977; Lohaus et al., 1994), respectively] must be energized. Apoplastic sucrose loading proceeds via a sucrose/H^+-symport (Giaquinta, 1977a,b; Komor et al., 1977; Delrot and Bonnemain, 1981; Bush, 1989) using the proton gradient which is established by the plasma membrane-located P-type H^+-ATPase. Direct evidence for an apoplastic step in sucrose loading into the phloem was provided by the expression of a yeast invertase in the apoplast (cell wall) of *Arabidopsis*, tobacco, tomato, and potato that led to increased carbohydrate levels in mature leaves and a concomitant decrease of photosynthesis (von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991; Heineke et al., 1992) resulting in reduced growth rates. These experiments demonstrated that only sucrose but not hexoses can be loaded into the phloem. The respective SUC2/SUT1 H^+-cotransporters have been identified by functional complementation of yeast strains unable to grow on sucrose (Riesmeier et al., 1992, 1993b; Sauer and Stolz, 1994). Expression of these transporters was confined to the phloem (Riesmeier et al., 1993b; Gahrtz et al., 1994; Truernit and Sauer, 1995), and more precisely to companion cells (Stadler et al., 1995; Stadler and Sauer, 1996; Schmitt et al., 2008). Functional proof was established by antisense down-regulation of the SUT1 proteins from potato (Riesmeier et al., 1994; Kühn et al., 1996) and tobacco (Bürkle et al., 1998). Knock-out *suc2 Arabidopsis* (Gottwald et al., 2000) and *sut1* maize (Slewinski et al., 2009, 2010) plants were characterized and displayed a similar phenotype to that found for the Solanaceae: carbohydrate accumulation in leaves, impaired growth and generally poor development of sinks. Over expression of a modified spinach SUT1 in potato did not lead to increased tuber starch content and yield (Leggewie et al., 2003). This could either be due to the use of a constitutive promoter to drive expression of the *SUT1* gene in mesophyll and phloem parenchyma cells that might have led to an unwanted re-uptake of sucrose from the apoplast into these cells and thus partial prevention of phloem loading. On the other hand, SUT1 activity might not be limiting for sucrose delivery to sink tissues or that potato tuber yield is not source-limited. *Arabidopsis suc2* mutants have been successfully complemented with the *Arabidopsis SUC1* gene expressed under control of the *AtSUC2* promoter. Some of these complemented lines displayed decreased leaf sucrose content indicative of more effective sucrose loading into the phloem (Wippel and Sauer, 2012). However, a possible impact on source capacity remains ambiguous since neither carbon export nor seed yield was comparatively analyzed in these plants. By over- and underexpressing the vacuolar TMT in *Arabidopsis* leaves, Wingenter et al. (2010) posed an indirect effect on *SUC2* transcripts, sucrose export from leaves and seed yield which have been found to positively correlate with TMT activity. However, it has to be elucidated whether or not TMT or other factors affect SUC2 activity which would in turn be accountable for altered source capacity.

LEAKAGE FROM AND RETRIEVAL OF SUCROSE TRANSPORTED IN THE PHLOEM

Sucrose in the phloem is transported to the sink tissues from the leaves and partially leaks out of the phloem to nourish the surrounding tissue (Minchin et al., 1984; Minchin and Thorpe, 1987). Whereas the above-ground tissues are green and might be able to support themselves to a certain extent by photosynthesis, the below-ground tissues clearly depend on sucrose leakage out of the phloem. How and where sucrose leaks out of the phloem has not been analyzed in detail. The sieve element/companion cell complex seems to be symplastically isolated (Kempers et al., 1998) along the whole path of sucrose movement indicating that unloading from the sieve elements and/or companion cells into the apoplast has to be facilitated. It remains to be elucidated whether SWEETs ("7" in **Figure 1**) are expressed in sieve elements or companion cells along the phloem and are responsible for this function.

Having entered the apoplast, sucrose then has to be taken up into the symplast again to reach the sink cells that depend on sucrose import. On the other hand, a great proportion of the sucrose that leaked out is retrieved into the sieve element/companion cell complex (Hafke et al., 2005). In both directions, uptake must be energized (Ayre, 2011), and thus sucrose/H^+-cotransporters are likely to fulfill this function. In *Arabidopsis*, the role of SUC2 ("8" in **Figure 1**) in retrieval of sucrose from the apoplast has been described by complementing the *suc2* mutant with a construct that restored SUC2 function in phloem loading (see above) but not in retrieval. The corresponding plants were smaller and exuded less ^{14}C label from cut petioles when leaves photosynthesized in the presence of $^{14}CO_2$. These findings are consistent with a role of SUC2 in retrieval of sucrose from the apoplast along the phloem (Srivastava et al., 2008). Analyses using ^{11}C tracer studies support this role for AtSUC2 (Gould et al., 2012). In addition, localization in the sieve elements of *Arabidopsis* stems argues for a putative role of SUC3 in retrieval ("9" in **Figure 1**; Meyer et al., 2000, 2004). This is in addition to SUC2 which seems to retrieve sucrose back into companion cells. However, it remains unclear which sucrose/H^+-cotransporters function in taking up sucrose to nourish the tissue surrounding the vasculature.

CARBON UNLOADING AND STORAGE IN SINK ORGANS

Unloading of sucrose from the phloem can occur symplastically or apoplastically, depending not only on the plant species but also on tissue types and developmental stages.

OIL STORAGE IN PLANTS

Most of the research on plants that store oil in their seed was performed on *Arabidopsis thaliana*. The important seed oil crop canola with cultivars of rapeseed (*Brassica napus*) or field mustard (*Brassica rapa*) belongs to the same family as *Arabidopsis*, the Brassicaceae. However, other plants with oil-storing seeds belong to the Asteraceae family, e.g., sunflower (*Helianthus annuus*), to

the Fabaceae family, e.g., soybean (*Glycine max*), or the Arecaceae family, e.g., oil palms (*Elaeis guineensis*, *Elaeis oleifera*, *Attalea maripa*). Oil palms not only store oil in their seeds but also in their fruits.

However, sucrose delivered from source leaves via the phloem has to reach the terminal storage sinks, i.e., seeds and/or fruits, thereby crossing several membranes. Once there, sucrose undergoes a series of metabolic reactions to be stored as triacylglycerol (TAG) in the embryo again including several transport steps of intermediates across membranes. We will focus on *Arabidopsis* to describe the path of carbon from sucrose to TAG in the following paragraphs.

To define the symplastic domains, mobile and immobile versions of the GFP under the control of a variety of promoters as well as the low-molecular-weight fluorescent dye HPTS (8-hydroxypyrene-1,3,6-trisulfonate) were used (Schneidereit et al., 2003; Stadler et al., 2005a).

Having reached the funicular end of the phloem, sucrose is unloaded into an unloading domain near the funiculus, and post-phloem transport occurs symplastically into the seed coat (Imlau et al., 1999), or – more precisely – into the outer integument (Stadler et al., 2005b). Before anthesis, phloem unloading of sucrose switches from symplastic in ovule primordia cells to apoplastic in mature ovules and upon anthesis back to a symplastic mode (Werner et al., 2011). To progress into the inner integument, sucrose must reach the apoplast which is potentially facilitated by SWEETs. A sucrose/H^+-symporter could also potentially transport sucrose into the inner integument. SUC3 has been discussed as a likely candidate (Stadler et al., 2005b) since it might be expressed in this cell layer (Meyer et al., 2004). However, the transfer of sucrose from the outer into the inner integument has not been analyzed in detail. The three layers of the inner integument form a symplastic continuum (Stadler et al., 2005b) isolated from the endosperm. After export from the inner integument, possibly mediated by a SWEET transporter, sucrose could be taken up into the endosperm by SUC5 which is expressed during seed development (Baud et al., 2005; Stadler et al., 2005b; Pommerrenig et al., 2013). Finally, before entering the embryo, sucrose has to be released into the apoplast separating endosperm and embryo. Again, one can speculate of a SWEET transporter family member ("7" in **Figure 2**) facilitating diffusion of sucrose out of the endosperm. Sucrose uptake into the embryo could be managed by three different SUC proteins (SUC3, -5, -9; **Figure 2**) that are expressed in different embryonic tissues at different developmental stages. With the help of a SUC9 promoter–GUS fusion, SUC9 expression could be detected in the mature embryo (Sivitz et al., 2007). More detailed analyses have been performed for the expression of both SUC5 and SUC3. In addition to the expression of SUC5 in the endosperm, torpedo and walking stick stage embryos express SUC5 in the epidermis of cotyledons (Pommerrenig et al., 2013). SUC3 is expressed in the suspensor which forms a symplastic continuum with the globular embryo. However, as early as the heart stage, the embryo proper is again symplastically isolated from the suspensor (Stadler et al., 2005b). From the torpedo stage on, SUC3 is expressed in the root epidermis which forms a symplastic connection with root and hypocotyls cells (Meyer et al., 2004; Stadler et al., 2005b). In almost fully developed embryos, SUC3 is still expressed in the root epidermis but seems to form a symplast with cells of the developing stele. Moreover, individual symplastically isolated cells of cotyledons express SUC3 (Stadler et al., 2005b). However, there seems to be redundancy in the described steps since none of the individual mutants displayed a phenotype different from wild-type. Whereas, there was no aberrant *suc3* phenotype detectable at all (Barth et al., 2003), *suc5* mutants displayed a transient decrease in seed TAG accumulation (Baud et al., 2005) which might be explained by the biotin transport function of SUC5 rather than the sucrose transport function (Pommerrenig et al., 2013). Under short-day conditions, *suc9* mutants show an early flowering phenotype but normally are indistinguishable from wild-type (Sivitz et al., 2007).

Once having reached the cytosol of embryo cells, sucrose is utilized to keep up metabolism and ultimately to build up TAG. TAG is formed from glycerol 3-phosphate (Gly3P) and acylated fatty acids in the endoplasmic reticulum (ER) in a series of reactions known as the Kennedy pathway (Kennedy, 1961). In plants, fatty acid synthesis takes place exclusively in plastids, and in heterotrophic tissues plastids depend on the import of carbon skeletons, energy and reduction power (Rawsthorne, 2002). Fatty acid synthesis starts from pyruvate that is first converted to acetyl-CoA by the plastidic pyruvate dehydrogenase complex. For synthesizing fatty acids from acetyl-CoA, ATP and NAD(P)H are required and thus have to be imported or generated within plastids. Carbon skeletons can be imported into plastids as glucose 6-phosphate (Glc6P), pyruvate, phosphoenolpyruvate (PEP), or malate. These metabolites are either intermediates of glycolysis, or in the case of malate, a tricarboxylic acid (TCA) cycle intermediate that can be exported from the mitochondria into the cytosol.

Glucose 6-phosphate is imported into plastids by the Glc6P/phosphate translocator (GPT; "10" in **Figure 2**; Kammerer et al., 1998) in counter-exchange with inorganic phosphate or triose phosphate generated by the oxidative pentose phosphate pathway (OPPP). Plastids isolated from young oilseed rape embryos utilize Glc6P better than pyruvate for fatty acid synthesis whereas plastids isolated from older embryos prefer pyruvate over Glc6P as a substrate for fatty acid synthesis (Eastmond and Rawsthorne, 2000). Moreover, imported Glc6P is also used for starch synthesis or as a substrate of the OPPP which is necessary for the production of NADPH required for fatty acid synthesis. Furthermore, partitioning of Glc6P into the different pathways is dependent on the developmental stage of the embryos (Eastmond and Rawsthorne, 2000). Seeds of *Arabidopsis* and oilseed rape transiently accumulate starch but completely degrade it until they reach maturity (da Silva et al., 1997; Baud et al., 2002; Andriotis et al., 2010a). Starch does not accumulate because of photosynthetic activity of the embryo and/or the seed coat, since seeds developing in darkened siliques also show transient starch accumulation (da Silva et al., 1997). It might be assumed that transient starch accumulation of seeds could be important for final TAG yield of embryos since *Arabidopsis* mutants defective in starch degradation (*sex1*; Yu et al., 2001) or biosynthesis (*phosphoglucomutase 1*, *pgm1*; Caspar et al., 1985) were found to contain 30 (Andriotis et al., 2010a) and 40% (Periappuram et al., 2000) less lipid content, respectively. In oilseed rape, the embryo-specific reduction of ADP-glucose pyrophosphorylase (AGPase) activity,

i.e., down-regulation of starch synthesis, led to a delay in oil accumulation of developing seeds. However, mature seeds had wild-type seed oil content (Vigeolas et al., 2004). Andriotis et al. (2012) were able to show that starch turnover of the maternal plant is important for *Arabidopsis* seed TAG yield, and not starch turnover of the embryo itself, i.e., they found a reduction in (mainly nocturnal) source capacity causative for compromised seed TAG content. Nonetheless, Glc6P uptake and partitioning into fatty acid and the OPPP seems to be of major importance since restricting GPT activity by seed-specific antisense expression or RNA interference (RNAi) led to an arrest of embryo development despite the redundancy of carbon skeleton uptake systems of plastids. As mentioned above, pyruvate as a direct substrate for fatty acid synthesis can also be taken up by plastids. The plastid sodium-dependent pyruvate transporter BASS2 was found to import pyruvate into plastids ("11" in **Figure 2**; Furumoto et al., 2011). Alternatively, pyruvate can be produced from PEP that could either be formed within the plastid stroma (Flügge et al., 2011) or imported by the PEP/phosphate translocator (PPT; "12" in **Figure 2**; Fischer et al., 1997). Moreover, malate can also serve as a precursor for stromal pyruvate. NADP malic enzyme produces pyruvate, CO_2 and reduction power from malate that could be imported into plastids by the dicarboxylate transporter DiT2 ("13" in **Figure 2**; Renné et al., 2003). However, none of these substrates can fuel the OPPP. Therefore, compromised generation of sufficient reduction power might explain the severity of the GPT antisense or RNAi plant phenotype (Andriotis et al., 2010b). Whereas, in *bass2* mutants there was no aberrant phenotype under normal growth conditions, though these were not analyzed for fatty acid or seed TAG content (Furumoto et al., 2011), *ppt1* (*cue1*; Li et al., 1995) mutants display a reticulate phenotype that spoils meaningful yield analyses. The supposed reason for the apparent phenotype is that PPT1 is not only involved in substrate supply for fatty acid synthesis but also important in secondary metabolite production (Streatfield et al., 1999; Voll et al., 2003).

DiT2 is essential for photorespiration, in that, *dit2* (*dct*; Somerville and Ogren, 1983; Somerville and Somerville, 1985) mutants do not re-assimilate ammonium released during photorespiration due to a lack of glutamate export from plastids and do not survive when grown under ambient CO_2 concentrations.

As mentioned above, energy has to be imported into heterotrophic plastids to drive – amongst other processes – transient starch synthesis and optimal fatty acid synthesis. Plastids import ATP via adenylate translocators (NTTs; "14" in **Figure 2**;

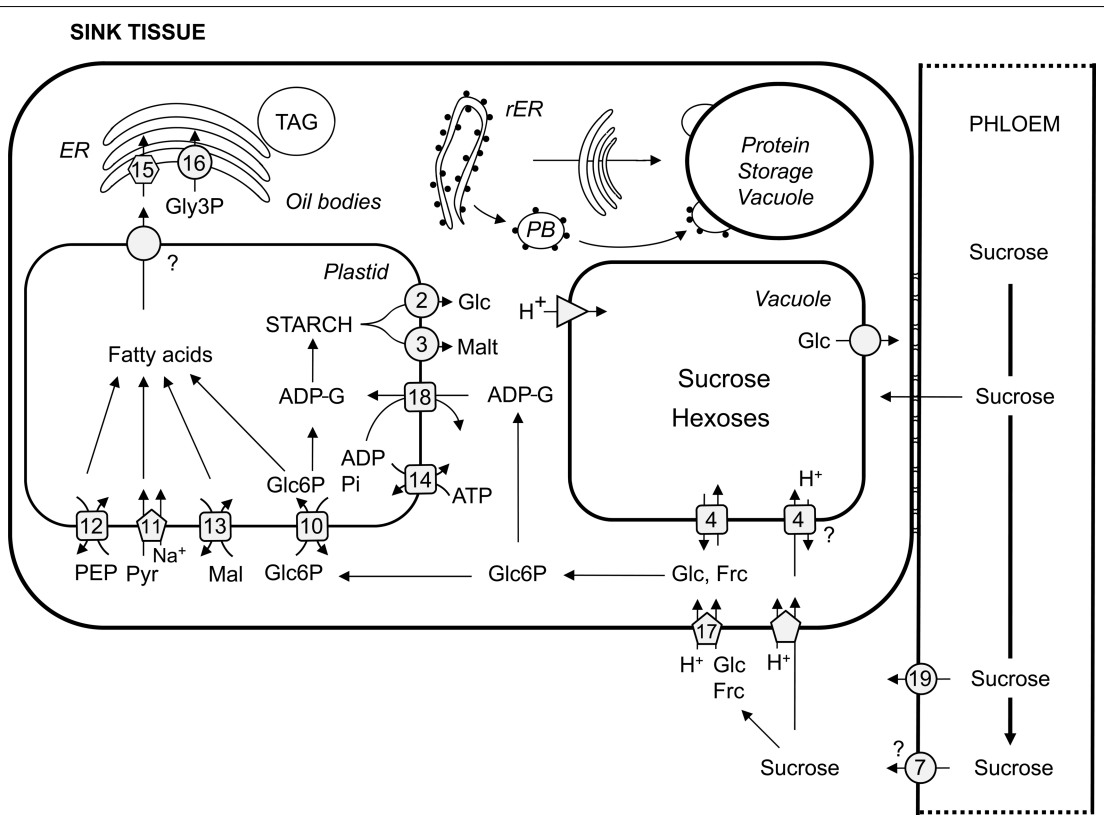

FIGURE 2 | Overview of carbon transport proteins in sink organs. Transport steps in sieve elements and sink cells are depicted according to their respective mode of action. Squares represent antiporters, circles describe facilitators, pentagons depict symporters, hexagons represent ABC transporters, and triangles H^+-ATPases/PPases. 2, pGlcT; 3, MEX; 4, TMT and VGT; 7, SWEETs; 10, GPT; 11, BASS2; 12, PPT, 13, DiT2; 14, NTT; 15, ABCA9; 16, Gly3P permease; 17, HT; 18, BT-1; 19, SUF1. (r)ER, (rough) endoplasmic reticulum; TAG, triacylglycerol; PB, protein body; PEP, phosphoenolpyruvate; Pyr, pyruvate; Mal, malate; Glc6P, glucose 6-phosphate; Pi, orthophosphate; ADP, adenosine diphosphate; ADP-G, ADP-glucose; Glc, glucose; Malt, maltose; Frc, fructose. Please refer to text for transporter abbreviations.

Kampfenkel et al., 1995) in counter-exchange with ADP and orthophosphate (Trentmann et al., 2008). *Arabidopsis* contains two NTTs and indeed *ntt1/ntt2* double mutants are impaired in seed protein and lipid contents and in seed yield (Reiser et al., 2004).

Fatty acids up to a length of 18 carbon atoms are produced in plastids. Incorporation of acylated fatty acids into TAG occurs in the ER (Li-Beisson et al., 2013). Hence, fatty acids have to be exported from plastids presumably by the recently identified fatty acid exporter FAX1 (K. Philippar, personal communication), and imported into the ER, and prior to incorporation into TAG have to be acylated by LACS proteins at the plastid outer envelope membrane (Shockey et al., 2002). To prevent inhibition of plastid metabolite transporters and thus fatty acid synthesis by cytosolic acyl-CoAs (Fox et al., 2001) and to assure efficient TAG production from acyl-CoAs, uptake into the ER should be rapid. Often reported spatial proximity of plastids or plastid stromules (filamentous tubules increasing the plastid surface area) and the ER (Schattat et al., 2011) might support a short retention time of acyl-CoA molecules in the cytosol. Recently, a transporter taking up free and acylated fatty acids into the ER has been described from *Arabidopsis*. The ABC transporter ABCA9 ("15" in **Figure 2**) has been found to localize to the ER, *abca9* mutants had decreased seed weight, total lipid and TAG and were defective in effectively taking up ^{14}C-labeled oleoyl-CoA and oleic acid. Moreover, constitutive overexpression of *ABCA9* driven by a constitutive promoter led to enlarged seeds with increased dry weight. The TAG content per seed was increased by 40% compared to wild-type. Since silique number per plant and seed number per silique were similar, overexpression of ABCA9 increased total seed oil per plant (Kim et al., 2013). This finding strongly argues for *Arabidopsis* yield to be sink-limited as it seems unlikely that the present expression of ABCA9 in leaves has a positive effect on source capacity. Moreover, it demonstrates that TAG production in the ER of embryo cells is limited by the supply with fatty acids rather than the Gly3P moiety of TAG. Nonetheless, Gly3P has to be imported into or present in the ER for TAG synthesis. This might be achieved by a member of the Gly3P permease family ("16" in **Figure 2**; Ramaiah et al., 2011), by phosphorylation of glycerol and/or by reduction of dihydroxyacetone phosphate. After having passed through the Kennedy pathway, synthesized TAG is stored in oil bodies (Hsieh and Huang, 2004; He and Wu, 2009) and can be used to fuel seedling establishment upon germination.

SUGAR STORAGE IN PLANTS

Some crop plants store sugars in tap roots, stems, and fruits, among them are sugar beet (*Beta vulgaris*), a member of the Amaranthaceae family, sugarcane (*Saccharum* hybrids) belonging to the Poaceae, grape (*Vitis vinifera*), a member of the Vitaceae family, and tomato (*Solanum lycopersicum*) belonging to the Solanaceae family. The former two species store sucrose whereas the latter two mainly store hexoses in vacuoles of roots, storage parenchyma, and fruit cells. Sucrose unloaded from the phloem has to reach these cells, and upon presence of an apoplastic step has to be imported into the cells either as sucrose or, when cleaved by an apoplastic invertase, as hexoses. Moreover, tonoplast transporters have to import sugars into the vacuole.

In young sugar beet tap roots growing with a rapid relative growth rate, sucrose unloaded from the phloem is cleaved by extracellular invertases indicating an apoplastic step in post-phloem transport. In older tap roots, invertase activities decrease and sucrose synthase activity increases (Klotz and Finger, 2002; Godt and Roitsch, 2006). Moreover, in experiments with ^{14}C-labeled sucrose fed to mature tap root tissue, there was little evidence for a hydrolytic step preceding sucrose uptake. Furthermore, the uptake of sucrose occurs against a concentration gradient, and thus requires metabolic energy (Giaquinta, 1979; Wyse, 1979). Vacuolar ATPase inhibitors prevented uptake of sucrose whereas plasma membrane ATPase inhibitors did not (Saftner et al., 1983) indicating that post-phloem sucrose transport into storage cells of mature tap roots occurs symplastically whereas import into the vacuole is energized by a V-type H^+-ATPase. This view is supported by analyses of tonoplast vesicles prepared from red beet root vacuoles which were found to hydrolyze ATP during sucrose transport (Getz, 1991). Sucrose-induced proton export occurred in a 1:1 stoichiometry (Getz and Klein, 1995) consistent with the idea of a sucrose/H^+-antiporter mediating the import of sucrose into storage vacuoles of sugar beet tap roots. Chiou and Bush (1996) described a tonoplast-localized transport protein expressed in leaves and tap roots. However, this transporter turned out to be a homolog of ERDL6, a vacuolar glucose exporter described above (**Figure 2**; Poschet et al., 2011).

In sugarcane, repeated sucrose breakdown and re-synthesis before storage in stem parenchyma cells has been described (Whittaker and Botha, 1997; Zhu et al., 1997). This "futile cycling" decreases with tissue maturity (Bindon and Botha, 2002; Uys et al., 2007). In mature internodes of stems sucrose seems to be symplastically unloaded since stem parenchyma cells are separated from the phloem by lignified and suberized cells preventing apoplastic unloading. Movement of the fluorescent tracer dye carboxyfluorescein from phloem to stem parenchyma cells indeed indicates symplastic connections. In younger internodes, a SUC2 ortholog is expressed in cells that are destined to be lignified (Rae et al., 2005) indicating an apoplastic step of sucrose unloading at this developmental stage. In mature internodes, sucrose was modeled to accumulate in vacuoles against a concentration gradient (Uys et al., 2007). This view was further substantiated by the detection of specialized acidic vacuoles with V-type H^+-ATPases in the stem (Rae et al., 2009), presumably maintaining the proton gradient that might be necessary to accumulate sucrose (against a concentration gradient) in a suggested proton antiport manner ("4" in **Figure 2**; Grof and Campbell, 2001). Both sugar beet and sugarcane have not been functionally analyzed regarding yield limitation, probably partially due to their resistance to be efficiently transformed.

Grape berries also undergo a developmental switch in unloading and post-phloem transport of sucrose. In contrast to sucrose-storing sugar beet and sugarcane, they shift from symplastic to apoplastic sucrose unloading upon onset of ripening as revealed by fluorescent dye analyses. Cell wall invertase activity increases at the onset of ripening, further substantiating apoplastic unloading at this developmental stage (Zhang et al., 2006). Moreover, Fillion et al. (1999) found that the STP-type hexose transporter VvHT1 ("17" in **Figure 2**) is expressed during ripening indicating

that some of the sucrose might be apoplastically cleaved by cell wall invertases before being taken up into the cytosol. Sugars accumulate mainly as hexoses in vacuoles of berry cells upon onset of ripening (Davies and Robinson, 1996). In a comprehensive approach, Afoufa-Bastien et al. (2010) gave a phylogenetic overview on sugar transporters and also analyzed their expression. Upon the onset of ripening, several hexose transporters, TMTs and SUC11/12 were expressed in berries. SUC11/12 expression in ripening berries was also found in an independent study (Manning et al., 2001), and VvTMT2 ("4" in **Figure 2**) expression has also been identified as ripening-related by Cakir and Giachino (2012). Taken together, expression analyses of several transporters support the finding that vacuolar storage of hexoses occurs by a switch to apoplastic unloading of sucrose upon onset of berry ripening. However, whether source- or sink capacity determines yield of grape berries remains elusive.

Tomato fruits and grape berries behave similarly with respect to sucrose unloading and post-phloem transport. In young tomato fruits, when starch content is built up in plastids of the columella and inner pericarp region of the fruit (Robinson et al., 1988; Schaffer and Petreikov, 1997), unloading is mainly symplastic. Upon the developmental shift to rapid hexose accumulation, also in the outer pericarp region, an apoplastic step is likely to occur (Ruan and Patrick, 1995) and is linked to an energized uptake of hexoses ("17" in **Figure 2**; Damon et al., 1988; Ruan et al., 1997) and sucrose (**Figure 2**; Damon et al., 1988). Similar to sugarcane, "futile cycles" involving invertase, sucrose synthase and starch synthesis and degradation seem to be abundant in tomato fruits to sustain sink capacity (Nguyen-Quoc and Foyer, 2001). The importance of transient starch accumulation for yield – unlike the above mentioned Brassicaceae seeds – was demonstrated by Baxter et al. (2005) who analyzed an introgression line with a fruit apoplastic invertase from the wild species *Solanum pennellii* introgressed into *Solanum lycopersicum*. Here, a dramatic increase in starch accumulation in early developmental stages was observed pointing toward partial apoplastic unloading already at younger developmental stages. Introgression lines had higher total soluble solids (TSS), a major determinant of fruit quality for processing (Baxter et al., 2005). More direct evidence for the importance of transient starch accumulation has been obtained by Petreikov et al. (2009) who developed and characterized introgression lines of *Solanum lycopersicum* harboring a wild species *Solanum habrochaites* allele for the regulatory large subunit of AGPase. This led to a large transient increase in starch which accounted for the enhanced amount of TSS and also an increase in fruit size. However, when hexose transporter- (STP-type) mediated uptake of hexoses from the apoplast was impaired by antisense expression of tomato *HT1*, *HT2*, and *HT3* ("17" in **Figure 2**; McCurdy et al., 2010), yield was decreased. Taken together, these findings point toward tomato yield being limited by sink capacity that can be increased when limitations of photoassimilate import into fruits are abolished.

STARCH STORAGE IN PLANTS

Apart from soybean and sugarcane, the most important crops store starch, among them the grasses corn (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) belonging to the Poaceae family, and potato (*Solanum tuberosum*), a member of the Solanaceae family. Other starch-storing staple foods are yams (*Dioscorea* sp.), belonging to the Dioscoreaceae, or cassava (*Manihot esculenta*), a member of the Euphorbiaceae. Grasses store starch in the endosperm of their caryopses, popularly called the "grain." Potato, yams, and cassava store starch in tubers, the former tuber is derived from the sprout, the two latter tubers from roots.

In grasses, sucrose released from the phloem in grains symplastically reaches the maternal side of the maternal/filial interface, in wheat described as nucellar projection (Fisher and Cash-Clark, 2000; Patrick and Offler, 2001; Krishnan and Dayanandan, 2003). At the interface which appears as an endosperm cavity in wheat grains, cells can specialize on the maternal, as well as, on the filial side to transfer cells (characterized by cell wall in growths) to maximize plasma membrane size for release and uptake of sugars. In corn missing such an endosperm cavity, the filial cells can undergo specialization to transfer cells (Patrick and Offler, 2001; Gómez et al., 2002; Krishnan and Dayanandan, 2003). It can be hypothesized that sugar unloading is mediated by SWEETs ("7" in **Figure 2**). To enter the filial tissue, sugars have to be taken up from the apoplast, likely to be mediated by sucrose- or hexose/H^+-symporters (**Figure 2**). In the endosperm, symplastic connections between uptake and storage cells exist (Patrick and Offler, 2001).

In cereal endosperm, starch synthesis differs from other starch-storing organs in that most of the AGPase activity is extraplastidic, i.e., cytosolic (Denyer et al., 1996; Thorbjörnsen et al., 1996; Beckles et al., 2001; James et al., 2003), requiring an additional uptake system for carbon skeletons into plastids supplementary to the GPT ("10" in **Figure 2**; Kammerer et al., 1998). Externally supplied ADP-glucose (ADP-G) was found to drive starch synthesis in isolated maize endosperm amyloplasts (Möhlmann et al., 1997) while Shannon et al. (1998) found Brittle-1 (BT-1; "18" in **Figure 2**; Sullivan and Kaneko, 1995) to be responsible for the uptake of ADP-G into maize endosperm amyloplasts by including the *bt-1* mutant in the analyses. Direct uptake measurements with the BT-1 from maize endosperm heterologously expressed in *Escherichia coli* cells revealed ADP-G uptake in counter-exchange with ADP (Kirchberger et al., 2007). Several approaches to increase starch yield of wheat and corn grains were successful when cytosolic AGPase activity was increased (Smidansky et al., 2002; Wang et al., 2007; Li et al., 2011), indicating that ADP-G import and thus BT-1 activity might not be limiting for starch yield. However, in contrast to the notion that photosynthesis is the limiting factor for increased yield (e.g., Long et al., 2006; Makino, 2011), these results suggest that sink- rather than source capacity limits grain starch yield in cereals.

In potato, starch is stored in tubers from which the plant can vegetatively, i.e., clonally propagate without a filial generation. Tubers are formed from stolons, below-ground lateral shoots. Upon induction of tuberization (Rodríguez-Falcón et al., 2006), stolons start to swell at their apical hook. In non-tuberized stolons, sucrose transported in the phloem is unloaded apoplastically. This view is supported by experiments using carboxyfluorescein trapped in the phloem and ^{14}C-labeled assimilates, mainly sucrose, which were evenly distributed in the stolon, i.e., also outside the phloem. Upon tuberization, carboxyfluorescein was able to leave the phloem, but only in the swollen part of the

stolon, indicative for symplastic unloading. Taken together, tuberization involves a switch from apoplastic to symplastic phloem unloading (Viola et al., 2001). However, this switch is likely to be incomplete because otherwise an impact of apoplastically localized yeast invertase expressed under control of a tuber-specific promoter on metabolism could not be explained (Sonnewald et al., 1997; Hajirezaei et al., 2000). Alternatively, symplastically unloaded sucrose might leak out of sink cells. Again, a yet to be identified member of the SWEET family transporter ("7" in **Figure 2**) may be discussed as the facilitator of apoplastic phloem unloading or as the transporter mediating leakage of sucrose. Whether mainly sucrose or hexoses – produced from sucrose by cell wall invertases – are imported into sink cells probably by sucrose- or hexose/H^+-symporters (**Figure 2**) remains to be elucidated. It is also unknown which transporters are engaged in this process. Sucrose entering sink cells via the apoplast or plasmodesmata might be further metabolized by sucrose synthase, since invertase activity is gradually reduced, while sucrose synthase activity yielding UPG-glucose and fructose increased during development (Hajirezaei et al., 2000). A part of the imported sucrose is further used to drive energy production indispensable for anabolic processes such as starch synthesis in heterotrophic tuber tissue. To synthesize starch, carbon skeletons and energy are imported into amyloplasts. Glc6P is imported by a GPT ("10" in **Figure 2**) in counter-exchange with orthophosphate or triose phosphate, and ATP generated in mitochondria is imported by the adenylate translocator NTT ("14" in **Figure 2**) in counter-exchange with ADP and orthophosphate. Shortage of energy import into amyloplasts as revealed by antisense repression of the adenylate translocator, led to compromised tuber starch content and yield (Tjaden et al., 1998). Overexpression, however, had no impact on starch yield, irrespective of the promoter, constitutive (Tjaden et al., 1998) or tuber-specific (Zhang et al., 2008), that drove expression of the transgene. Tuber-specific overexpression of a pea GPT also had no impact on tuber starch yield. Only when both carbon and energy supply to plastids was increased simultaneously, mediated by overexpression of GPT and NTT, tuber starch content and yield increased (Zhang et al., 2008). This indicated that import of carbon and energy co-limit starch yield which appears sink- rather than source-limited under these growth conditions. Moreover, a combined enhancement of sink- and source capacities led to even higher starch yield of triple-transgenic potato plants. The overexpression of GPT and NTT in tubers and additionally either overexpression of an *Escherichia coli* pyrophosphatase in mesophyll cytosol or reduced AGPase activity in leaves increased sucrose synthesis in source tissues (Jonik et al., 2012). Without simultaneously increasing sink capacity, neither leaf-specific antisense repression of AGPase (Leidreiter et al., 1995) nor mesophyll-specific cytosolic overexpression of pyrophosphatase (Jonik et al., 2012) – the latter analyzed in field trials and in the greenhouse – led to an increase in tuber starch yield indicating once more that potato tuber starch yield is sink-limited.

PROTEIN STORAGE IN PLANTS

Members of the Fabaceae family store protein in their seeds, which represent the main plant source for human nitrogen nutrition. However, soybean (*G. max*) is primarily grown to provide oil from seeds, and pea (*Pisum sativum*) seeds contain more starch than protein. Other crop members of the family are bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), and peanut (*Arachis hypogaea*). There are species of families other than Fabaceae which contain reasonable amounts of storage protein in their harvested organs. However, these are mainly grown for their starch (e.g., potato, corn) or oil (e.g., canola). In order to store protein ample nitrogen must be available. Consistently, members of the Fabaceae family living in symbiosis with rhizobial bacteria store protein in seeds as the symbiotic bacteria fix atmospheric molecular nitrogen and supply the host plant with sufficient reduced nitrogen.

In legumes, protein is stored in the embryo in structures known as protein storage vacuoles. Storage proteins are translated at the ribosomes of the rough ER and are co-translationally imported into the ER. The main route from the ER to protein storage vacuoles is via the Golgi (Herman and Larkins, 1999). However, the alternative pathway, protein bodies budding off the ER and either remaining in the cytosol or fusing with the protein storage vacuole (as previously described in cereals) also seems to exist in legumes (**Figure 2**; Herman and Larkins, 1999; Vitale and Ceriotti, 2004; Abirached-Darmency et al., 2012).

Sucrose (and amino acid) unloading from the phloem in legume seeds occurs symplastically. Analyses with fluorescent dyes indicate that sucrose is symplastically transported to the maternal release site, the ground- or thin-walled parenchyma (Tegeder et al., 1999; van Dongen et al., 2003). Sucrose is either simply or through specialized transfer cells released into the apoplast dependent on the legume species (Patrick and Offler, 2001; van Dongen et al., 2003). At the younger stages, legume seeds contain a liquid endosperm which acts as a buffer for sucrose and glutamine for the developing embryo (Melkus et al., 2009). At the expense of embryo growth, the endosperm is substantially degraded during development (Patrick and Offler, 2001). At later developmental stages, sucrose is released from maternal tissue to the apoplast and is taken up by embryo epidermal transfer cells (Weber et al., 1997; Tegeder et al., 1999). With the discovery of a new class of sucrose facilitators from pea and bean, called SUFs, expressed at the maternal release site, sucrose release into the apoplast is probably mediated by these transporters ("19" in **Figure 2**; Zhou et al., 2007). In these same cells, the sucrose/H^+-symporter SUT1 was found to be expressed and believed to likely function in seed coat sucrose retrieval (Tegeder et al., 1999; Zhou et al., 2007). At the filial side, uptake of sucrose by the embryo epidermis is mediated by SUT1 (**Figure 2**), and uptake is energized by P-type H^+-ATPases which are both co-expressed in embryo transfer cells (Tegeder et al., 1999). The embryo communicates the demand for sucrose uptake from the apoplast by the internal sugar level. Sucrose uptake fluxes have been found to be negatively correlated with pool sizes of intracellular sugars, while *SUT1* transcripts were sensitive to sugar levels (i.e., when the embryo contains sufficient sugars, *SUT1* is transcriptionally down-regulated), which in turn led to decreased sucrose uptake (Zhou et al., 2009). To increase the uptake of sucrose into pea embryos, potato SUT1 was overexpressed using a storage parenchyma-specific promoter which resulted in increased sucrose uptake and accelerated growth rates of the embryo. However, final seed weight of transgenic plants was

similar to the wild-type. The authors speculate that overexpression at the primary site of sucrose uptake, the epidermal transfer cells, might have led to more significant results (Rosche et al., 2002). In a different transgenic approach, seed protein content in mature seeds was increased. Rolletschek et al. (2007) antisense inhibited the *Vicia narbonensis GPT1* ("10" in **Figure 2**) using a seed-specific promoter. As expected, flux of carbon into plastids and thus into starch and (mainly structural) lipids was found to be decreased. A simultaneous increase in seed protein, however, was not predictable. The authors explain this finding with increased expression of the amino acid permease *AAP1* in transgenic embryos potentially due to lower glutamine concentrations during the main protein storage phase. Glutamine was previously described to repress *AAP1* transcriptionally (Miranda et al., 2001). Indeed, when AAP1 was overexpressed in seeds of *Vicia narbonensis* and pea, sink strength for amino acids was increased and the amounts of total nitrogen and protein (mainly storage globulins) were increased (Rolletschek et al., 2005).

CONCLUDING REMARKS

This review on plant carbon allocation and transport unequivocally demonstrates the increasing importance of sub- and intercellular, as well as, long-distance transport processes. Given the impact of several of the described modifications on yield of various crop plants, we suggest continued work on several of the insinuated and not yet conceived transport steps within plants which frequently turn out to be the rate-limiting steps for the production of valuable compounds in storage sinks.

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REFERENCES

Abirached-Darmency, M., Dessaint, F., Benlicha, E., and Schneider, C. (2012). Biogenesis of protein bodies during vicilin accumulation in *Medicago truncatula* immature seeds. *BMC Res. Notes* 5:409. doi: 10.1186/1756-0500-5-409

Afoufa-Bastien, D., Medici, A., Jeauffre, J., Coutos-Thévenot, P., Lemoine, R., Atanassova, R., et al. (2010). The *Vitis vinifera* sugar transporter gene family: phylogenetic overview and macroarray expression profiling. *BMC Plant Biol.* 10:245. doi: 10.1186/1471-2229-10-245

Ainsworth, E. A., and Bush, D. R. (2011). Carbohydrate export from the leaf: a highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiol.* 155, 64–69. doi: 10.1104/pp.110.167684

Aluri, S., and Büttner, M. (2007). Identification and functional expression of the *Arabidopsis thaliana* vacuolar glucose transporter 1 and its role in seed germination and flowering. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2537–2542. doi: 10.1073/pnas.0610278104

Andriotis, V. M. E., Pike, M. J., Kular, B., Rawsthorne, S., and Smith, A. M. (2010a). Starch turnover in developing oilseed embryos. *New Phytol.* 187, 791–804. doi: 10.1111/j.1469-8137.2010.03311.x

Andriotis, V. M. E., Pike, M. J., Bunnewell, S., Hills, M. J., and Smith, A. M. (2010b). The plastidial glucose-6-phosphate/phosphate antiporter GPT1 is essential for morphogenesis in *Arabidopsis* embryos. *Plant J.* 64, 128–139. doi: 10.1111/j.1365-313X.2010.04313.x

Andriotis, V. M. E., Pike, M. J., Schwarz, S. L., Rawsthorne, S., Wang, T. L., and Smith, A. M. (2012). Altered starch turnover in the maternal plant has major effects on *Arabidopsis* fruit growth and seed composition. *Plant Physiol.* 160, 1175–1186. doi: 10.1104/pp.112.205062

Antony, G., Zhou, J., Huang, S., Li, T., Liu, B., White, F., et al. (2010). Rice xa13 recessive resistance to bacterial blight is defeated by induction of the disease susceptibility gene Os-11N3. *Plant Cell* 22, 3864–3876. doi: 10.1105/tpc.110.078964

Ayre, B. G. (2011). Membrane-transport systems for sucrose in relation to whole-plant carbon partitioning. *Mol. Plant* 4, 377–394. doi: 10.1093/mp/ssr014

Baker, R. F., Leacvh, K. A., and Braun, D. M. (2012). SWEET as sugar: new sucrose effluxers in plants. *Mol. Plant* 5, 766–768. doi: 10.1093/mp/SSS054

Barth, I., Meyer, S., and Sauer, N. (2003). PmSUC3: characterization of a SUT2/SUC3-type sucrose transporter from *Plantago major*. *Plant Cell* 15, 1375–1385. doi: 10.1105/tpc.010967

Baud, S., Boutin, J.-P., Miquel, M., Lepiniec, L., and Rochat, C. (2002). An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. *Plant Physiol. Biochem.* 40, 151–160. doi: 10.1016/S0981-9428(01)01350-X

Baud, S., Wuillème, S., Lemoine, R., Kronenberger, J., Caboche, M., Lepiniec, L., et al. (2005). The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in *Arabidopsis*. *Plant J.* 43, 824–836.

Baxter, C. J., Carrari, F., Bauke, A., Overy, S., Hill, S. A., Quick, P. W., et al. (2005). Fruit carbohydrate metabolism in an introgressed line of tomato with increased fruit soluble solids. *Plant Cell Physiol.* 46, 425–437. doi: 10.1093/pcp/pci040

Beckles, D. M., Smith, A. M., and ap Rees, T. (2001). A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. *Plant Physiol.* 125, 818–827. doi: 10.1104/pp.125.2.818

Bindon, K. A., and Botha, F. C. (2002). Carbon allocation to the insoluble fraction, respiration and triose-phosphate cycling in the sugarcane culm. *Physiol. Plant.* 116,12–19. doi: 10.1034/j.1399-3054.2002.1160102.x

Braun, D. M. (2012). SWEET! The pathway is complete. *Science* 335, 173–174. doi: 10.1126/science.1216828

Bürkle, L., Hibberd, J., Quick, W. P., Kühn, C., Hirner, B., and Frommer, W. B. (1998). The H+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. *Plant Physiol.* 118, 59–68.

Bush, D. R. (1989). Proton-coupled sucrose transport in plasmalemma vesicles isolated from sugar beet (*Beta vulgaris* L. cv Great Western) leaves. *Plant Physiol.* 89, 1318–1323. doi: 10.1104/pp.89.4.1318

Büttner, M. (2007). The monosaccharide transporter(-like) gene family in *Arabidopsis*. *FEBS Lett.* 581, 2318–2324.

Büttner, M. (2010). The *Arabidopsis* sugar transporter (AtSTP) family: an update. *Plant Biol. Suppl.* 1, 35–41. doi: 10.1111/j.1438-8677.2010.00383.x

Cakir, B., and Giachino, R. R. A. (2012). VvTMT2 encodes a putative tonoplast monosaccharide transporter expressed during grape berry (*Vitis vinifera* cv. Sultanine) ripening. *Plant Omics J.* 5, 576–583.

Caspar, T., Huber, S. C., and Somerville, C. (1985). Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. *Plant Physiol.* 79, 11–17. doi: 10.1104/pp.79.1.11

Chardon, F., Bedu, M., Calenge, F., Klemens, P. A. W., Spinner, L., Clement, G., et al. (2013). Leaf fructose content is controlled by the vacuolar transporter SWEET17 in *Arabidopsis*. *Curr. Biol.* 23, 697–702. doi: 10.1016/j.cub.2013.03.021

Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X.-Q., et al. (2010). Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468, 527–534. doi: 10.1038/nature09606

Chen, L.-Q., Qu, X.-Q., Hou, B.-H., Sosso, D., Osorio, S., Fernie, A. R., et al. (2012). Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* 335, 207–211. doi: 10.1126/science.1213351

Chincinska, I. A., Liesche, J., Krügel, U., Michalska, J., Geigenberger, P., Grimm, B., et al. (2008). Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response. *Plant Physiol.* 146, 515–528.

Chiou, T. J., and Bush, D. R. (1996). Molecular cloning, immunochemical localization to the vacuole, and expression in transgenic yeast and tobacco of a putative sugar transporter from sugar beet. *Plant Physiol.* 110, 511–520. doi: 10.1104/pp.110.2.511

Cho, M.-H., Jang, A., Bhoo, S. H., Jeon, J.-S., and Hahn, T.-R. (2012). Manipulation of triose phosphate/phosphate translocator

and cytosolic fructose-1,6-bisphosphatase, the key components in photosynthetic sucrose synthesis, enhance the source capacity of transgenic *Arabidopsis* plants. *Photosynth. Res.* 111, 261–268. doi: 10.1007/s11120-012-9720-2

Cho, M.-H., Lim, H., Shin, D. H., Jeon, J.-S., Bhoo, S. H., Park, Y.-I., et al. (2011). Role of plastidic glucose transporters in the export of starch degradation products from the chloroplasts in *Arabidopsis thaliana*. *New Phytol.* 190, 101–112. doi: 10.1111/j.1469-8137.2010.03580.x

Damon, S., Hewitt, J., Nieder, M., and Bennett, A. B. (1988). Sink metabolism in tomato fruit. II. Phloem unloading and sugar uptake. *Plant Physiol.* 87, 731–736. doi: 10.1104/pp.87.3.731

da Silva, P. M. F. R., Eastmond, P. J., Hill, L. M., Smith, A. M., and Rawsthorne, S. (1997). Starch metabolism in developing embryos of oilseed rape. *Planta* 203, 480–487. doi: 10.1007/s004250050217

Davies, C., and Robinson, S. P. (1996). Sugar accumulation in grape berries. Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. *Plant Physiol.* 111, 275–283. doi: 10.1104/pp.111.1.275

Delrot, S., and Bonnemain, J.-L. (1981). Involvement of protons as a substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. *Plant Physiol.* 67, 560–564. doi: 10.1104/pp.67.3.560

Denyer, K., Dunlap, F., Thorbjörnsen, T., Keeling, P., and Smith, A. M. (1996). The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol.* 112, 779–785.

Dickinson, C. D., Altabella, T., and Chrispeels, M. J. (1991). Slow-growth phenotype of transgenic tomato expressing apoplastic invertase. *Plant Physiol.* 95, 420–425. doi: 10.1104/pp.95.2.420

Eastmond, P. J., and Rawsthorne, S. (2000). Coordinate changes in carbon partitioning and plastidial metabolism during the development of oilseed rape embryos. *Plant Physiol.* 122, 767–774. doi: 10.1104/pp.122.3.767

Endler, A., Meyer, S., Schelbert, S., Schneider, T., Weschke, W., Peters, S. W., et al. (2006). Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. *Plant Physiol.* 141, 196–207. doi: 10.1104/pp.106.079533

Eom, J.-S., Cho, J.-I., Reinders, A., Lee, S.-W., Yoo, Y., Tuan, P. Q., et al. (2011). Impaired function of the tonoplast-localized sucrose transporter in rice, OsSUT2, limits the transport of vacuolar reserve sucrose and affects plant growth. *Plant Physiol.* 157, 109–119. doi: 10.1104/pp.111.176982

Fillion, L., Ageorges, A., Picaud, S., Coutos-Thévenot, P., Lemoine, R., Romieu, C., et al. (1999). Cloning and expression of a hexose transporter gene expressed during ripening of grape berry. *Plant Physiol.* 120, 1083–1093.

Fischer, K., Kammerer, B., Gutensohn, M., Arbinger, B., Weber, A., Häusler, R. E., et al. (1997). A new class of plastidic phosphate translocators: a putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. *Plant Cell* 9, 453–462.

Fisher, D. B., and Cash-Clark, C. E. (2000). Sieve tube unloading and post-phloem transport of fluorescent tracers and proteins injected into sieve tubes via severed aphid stylets. *Plant Physiol.* 123, 125–137. doi: 10.1104/pp.123.1.125

Fliege, R., Flügge, U.-I., Werdan, K., and Heldt, H. W. (1978). Specific transport of inorganic phosphate, 3-phosphoglycerate and triosephosphates across the inner membrane of the envelope in spinach chloroplasts. *Biochim. Biophys. Acta* 502, 232–247.

Flügge, U.-I., Fischer, K., Gross, A., Sebald, W., Lottspeich, F., and Eckerskorn, C. (1989). The triose phosphate-3-phosphoglycerate-phosphate translocator from spinach chloroplasts: nucleotide sequence of a full-length cDNA clone and import of the in vitro synthesized precursor protein into chloroplasts. *EMBO J.* 8, 39–46.

Flügge, U.-I., Häusler, R. E., Ludewig, F., and Gierth, M. (2011). The role of transporters in supplying energy to plant plastids. *J. Exp. Bot.* 62, 2381–2392. doi: 10.1093/jxb/erq361

Fondy, B. R., and Geiger, D. R. (1977). Sugar selectivity and other characteristics of phloem loading in *Beta vulgaris* L. *Plant Physiol.* 59, 953–960. doi: 10.1104/pp.59.5.953

Fox, S. R., Rawsthorne, S., and Hills, M. J. (2001). Fatty acid synthesis in pea root plastids is inhibited by the action of long-chain acyl-coenzyme as on metabolite transporters. *Plant Physiol.* 126, 1259–1265. doi: 10.1104/pp.126.3.1259

Furumoto, T., Yamaguchi, T., Ohshima-Ichie, Y., Nakamura, M., Tsuchida-Iwata, Y., Shimamura, M., et al. (2011). A plastidial sodium-dependent pyruvate transporter. *Nature* 476, 472–475. doi: 10.1038/nature10250

Gahrtz, M., Stolz, J., and Sauer, N. (1994). A phloem-specific sucrose-H+ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. *Plant J.* 6, 697–706. doi: 10.1046/j.1365-313X.1994.6050697.x

Getz, H. P. (1991). Sucrose transport in tonoplast vesicles of red beet roots is linked to ATP hydrolysis. *Planta* 185, 261–268. doi: 10.1007/BF00194069

Getz, H. P., and Klein, M. (1995). Characteristics of sucrose transport and sucrose-induced H+ transport on the tonoplast of red beet (*Beta vulgaris* L.) storage tissue. *Plant Physiol.* 107, 459–467.

Giaquinta, R. (1977a). Phloem loading of sucrose. pH dependency and selectivity. *Plant Physiol.* 59, 750–755. doi: 10.1104/pp.59.4.750

Giaquinta, R. (1977b). Possible role of pH gradient and membrane ATPase in the loading of sucrose into the sieve tubes. *Nature* 267, 369–370. doi: 10.1038/267369a0

Giaquinta, R. T. (1979). Sucrose translocation and storage in the sugar beet. *Plant Physiol.* 63, 828–832. doi: 10.1104/pp.63.5.828

Godt, D., and Roitsch, T. (2006). The developmental and organ specific expression of sucrose cleaving enzymes in sugar beet suggests a transition between apoplasmic and symplasmic phloem unloading in the tap roots. *Plant Physiol. Biochem.* 44, 656–665. doi: 10.1016/j.plaphy.2006.09.019

Gómez, E., Royo, J., Guo, Y., Thompson, R., and Hueros, G. (2002). Establishment of cereal endosperm expression domains: identification and properties of a maize transfer cell-specific transcription factor, ZmMRP-1. *Plant Cell* 14, 599–610.

Gottwald, J. R., Krysan, P. J., Young, J. C., Evert, R. F., and Sussman, M. R. (2000). Genetic evidence for the in planta role of phloem-specific plasma membrane sucrose transporters. *Proc. Natl. Acad. Sci.* 97, 13979–13984. doi: 10.1073/pnas.250473797

Gould, N., Thorpe, M. R., Pritchard, J., Christeller, J. T., Williams, L. E., Roeb, G., et al. (2012). AtSUC2 has a role for sucrose retrieval along the phloem pathway: evidence from carbon-11 tracer studies. *Plant Sci.* 188–189, 97–101. doi: 10.1016/j.plantsci.2011.12.018

Grof, C. P. L., and Campbell, J. A. (2001). Sugarcane sucrose metabolism: scope for molecular manipulation. *Aust. J. Plant Physiol.* 28, 1–12.

Hafke, J. B., van Amerongen, J.-K., Kelling, F., Furch, A. C. U., Gaupels, F., and van Bel, A. J. E. (2005). Thermodynamic battle for photosynthate acquisition between sieve tubes and adjoining parenchyma in transport phloem. *Plant Physiol.* 138, 1527–1537. doi: 10.1104/pp.104.058511

Hajirezaei, M.-R., Takahata, Y., Trethewey, R. N., Willmitzer, L., and Sonnewald, U. (2000). Impact of elevated cytosolic and apoplastic invertase activity on carbon metabolism during potato tuber development. *J. Exp. Bot.* 51, 439–445. doi: 10.1093/jexbot/51.suppl_1.439

Häusler, R. E., Schlieben, N. H., Nicolay, P., Fischer, K., Fischer, K. L., and Flügge, U.-I. (2000). Control of carbon partitioning and photosynthesis by the triose phosphate/phosphate translocator in transgenic tobacco plants (*Nicotiana tabacum* L.). I. Comparative physiological analysis of tobacco plants with antisense repression and overexpression of the triose phosphate/phosphate translocator. *Planta* 210, 371–382.

Häusler, R. E., Schlieben, N. H., Schulz, B., and Flügge, U.-I. (1998). Compensation of decreased triose phosphate/phosphate translocator activity by accelerated starch turnover and glucose transport in transgenic tobacco. *Planta* 204, 366–376.

He, Y.-Q., and Wu, Y. (2009). Oil body biogenesis during *Brassica napus* embryogenesis. *J. Integr. Plant Biol.* 51, 792–799. doi: 10.1111/j.1744-7909.2009.00851.x

Heineke, D., Kruse, A., Flügge, U.-I., Frommer, W. B., Riesmeier, J. W., Willmitzer, L., et al. (1994). Effect of antisense repression of the chloroplast triose-phosphate translocator on photosynthetic metabolism in transgenic potato plants. *Planta* 193, 174–180.

Heineke, D., Sonnewald, U., Büssis, D., Günter, G., Leidreiter, K., Wilke, I., et al. (1992). Apoplastic expression of yeast-derived invertase in potato: effects on photosynthesis, leaf solute composition, water relations, and tuber composition. *Plant Physiol.* 100, 301–308.

Herman, E. M., and Larkins, B. A. (1999). Protein storage bodies and vacuoles. *Plant Cell* 11, 601–613.

Hsieh, K., and Huang, A. H. C. (2004). Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. *Plant Physiol.* 136, 3427–3434. doi: 10.1104/pp.104.051060

Imlau, A., Truernit, E., and Sauer, N. (1999). Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell* 11, 309–322.

James, M. G., Denyer, K., and Myers, A. M. (2003). Starch synthesis in the cereal endosperm. *Curr. Opin. Plant Biol.* 6, 215–222. doi: 10.1016/S1369-5266(03)00042-6

Jonik, C., Sonnewald, U., Hajirezaei, M. R., Flügge, U.-I., and Ludewig, F. (2012). Simultaneous boosting of source and sink capacities doubles tuber starch yield of potato plants. *Plant Biotechnol. J.* 10, 1088–1098. doi: 10.1111/j.1467-7652.2012.00736.x

Kammerer, B., Fischer, K., Hilpert, B., Schubert, S., Gutensohn, M., Weber, A., et al. (1998). Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. *Plant Cell* 10,105–117.

Kampfenkel, K., Möhlmann, T., Batz, O., van Montagu, M., Inzé, D., and Neuhaus, H. E. (1995). Molecular characterization of an *Arabidopsis thaliana* cDNA encoding a novel putative adenylate translocator of higher plants. *FEBS Lett.* 374, 351–355.

Kempers, R., Ammerlaan, A., and van Bel, A. J. E. (1998). Symplasmic constriction and ultrastructural feature of the sieve element/companion cell complex in the transport phloem of apoplasmically and symplasmically phloem-loading species. *Plant Physiol.* 116, 271–278. doi: 10.1104/pp.116.1.271

Kennedy, E. P. (1961). Biosynthesis of complex lipids. *Fed. Proc.* 20, 934–940.

Kim, S., Yamaoka, Y., Ono, H., Kim, H., Shim, D., Maeshima, M., et al. (2013). AtABCA9 transporter supplies fatty acids for lipid synthesis to the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 110, 773–778. doi: 10.1073/pnas.1214159110

Kirchberger, S., Leroch, M., Huynen, M. A., Wahl, M., Neuhaus, H. E., and Tjaden, J. (2007). Molecular and biochemical analysis of the plastidic ADP-glucose transporter (ZmBT1) from *Zea mays*. *J. Biol. Chem.* 282, 22481–22491. doi: 10.1074/jbc.M702484200

Klotz, K. L., and Finger, F. L. (2002). Contribution of invertase and sucrose synthase isoforms to sucrose catabolism in developing sugarbeet roots. *J. Sugar Beet Res.* 39, 1–24. doi: 10.5274/jsbr.39.1.1

Komor, E., Rotter, M., and Tanner, W. (1977). A proton-cotransport system in a higher plant: sucrose transport in *Ricinus communis*. *Plant Sci. Lett.* 9, 153–162. doi: 10.1016/0304-4211(77)90093-1

Krishnan, S., and Dayanandan, P. (2003). Structural and histochemical studies on grain-filling in the caryopsis of rice (*Oryza sativa* L.). *J. Biosci.* 28, 455–469. doi: 10.1007/BF02705120

Kühn, C., and Grof, C. P. L. (2010). Sucrose transporters of higher plants. *Curr. Opin. Plant Biol.* 13, 288–298. doi: 10.1016/j.pbi.2010.02.001

Kühn, C., Quick, W. P., Schulz, A., Sonnewald, U., and Frommer, W. B. (1996). Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ.* 19, 1115–1123. doi: 10.1111/j.1365-3040.1996.tb00426.x

Leggewie, G., Kolbe, A., Lemoine, R., Roessner, U., Lytovchenko, A., Zuther, E., et al. (2003). Overexpression of the sucrose transporter SoSUT1 in potato results in alterations in leaf carbon partitioning and in tuber metabolism but has little impact on tuber morphology. *Planta* 217, 158–167.

Leidreiter, K., Heineke, D., Heldt, H. W., Müller-Röber, B., Sonnewald, U., and Willmitzer, L. (1995). Leaf-specific antisense inhibition of starch biosynthesis in transgenic potato plants leads to an increase in photoassimilate export from source leaves during the light period. *Plant Cell Physiol.* 36, 615–624.

Li, H. M., Culligan, K., Dixon, R. A., and Chory, J. (1995). CUE1: a mesophyll cell-specific positive regulator of light-controlled gene expression in *Arabidopsis*. *Plant Cell* 7, 1599–1610.

Li, N., Zhang, S., Zhao, Y., Li, B., and Zhang, J. (2011). Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. *Planta* 233, 241–250. doi: 10.1007/s00425-010-1296-5

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andresson, M. X., Arondel, V., Bates, P. D., et al. (2013). Acyl-lipid metabolism. *Arabidopsis Book* 11:e0161. doi: 10.1199/tab.0161

Lohaus, G., Burba, M., and Heldt, H. W. (1994). Comparison of the contents of sucrose and amino acids in the leaves, phloem sap and taproots of high and low sugar-producing hybrids of sugar beet (*Beta vulgaris* L.). *J. Exp. Bot.* 45, 1097–1101. doi: 10.1093/jxb/45.8.1097

Lohaus, G., Winter, H., Riens, B., and Heldt, H. W. (1995). Further studies on the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in sieve tubes. *Bot. Acta* 108, 270–275.

Long, S. P., Zhu, X.-G., Naidu, S. L., and Ort, D. R. (2006). Can improvement in photosynthesis increase crop yields? *Plant Cell Environ.* 29, 315–330. doi: 10.1111/j.1365-3040.2005.01493.x

Makino, A. (2011). Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. *Plant Physiol.* 155, 125–129. doi: 10.1104/pp.110.165076

Manning, K., Davies, C., Bowen, H. C., and White, P. J. (2001). Functional characterization of two ripening-related sucrose transporters from grape berries. *Ann. Bot.* 87, 125–129. doi: 10.1006/anbo.2000.1316

Martinoia, E., Massonneau, A., and Frangne, N. (2000). Transport processes of solutes across the vacuolar membrane of higher plants. *Plant Cell Physiol.* 41, 1175–1186. doi: 10.1093/pcp/pcd059

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2006). Sink strength regulates photosynthesis in sugarcane. *New Phytol.* 171, 759–770. doi: 10.1111/j.1469-8137.2006.01785.x

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2008). Changes in photosynthetic rates and gene expression of leaves during a source–sink perturbation in sugarcane. *Ann. Bot.* 101, 89–102. doi: 10.1093/aob/mcm258

McCouch, S. (2004). Diversifying selection in plant breeding. *PLoS Biol.* 2:e347. doi: 10.1371/journal.pbio.0020347

McCurdy, D. W., Dibley, S., Cahyanegara, R., Martin, A., and Patrick, J. W. (2010). Functional characterization and RNAi-mediated suppression reveals roles for hexose transporters in sugar accumulation by tomato fruit. *Mol. Plant* 3, 1049–1063. doi: 10.1093/mp/ssq050

Melkus, G., Rolletschek, H., Radchuk, R., Fuchs, J., Rutten, T., Wobus, U., et al. (2009). The metabolic role of the legume endosperm: a noninvasive imaging study. *Plant Physiol.* 151, 1139–1154. doi: 10.1104/pp.109.143974

Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R. D., and Sauer, N. (2004). Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. *Plant Physiol.* 134, 684–693. doi: 10.1104/pp.103.033399

Meyer, S., Melzer, M., Truernit, E., Hümmer, C., Besenbeck, R., Stadler, R., et al. (2000). AtSUC3, a gene encoding a new *Arabidopsis* sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. *Plant J.* 24, 869–882.

Minchin, P. E. H., Ryan, K. G., and Thorpe, M. R. (1984). Further evidence of apoplastic unloading into the stem of bean: identification of the phloem buffering pool. *J. Exp. Bot.* 35, 1744–1753. doi: 10.1093/jxb/35.12.1744

Minchin, P. E. H., and Thorpe, M. R. (1987). Measurement of unloading and reloading of photo-assimilate within the stem of bean. *J. Exp. Bot.* 38, 211–220. doi: 10.1093/jxb/38.2.211

Miranda, M., Borisjuk, L., Tewes, A., Heim, U., Sauer, N., Wobus, U., et al. (2001). Amino acid permeases in developing seeds of *Vicia faba* L.: expression precedes storage protein synthesis and is regulated by amino acid supply. *Plant J.* 28, 61–72. doi: 10.1046/j.1365-313X.2001.01129.x

Möhlmann, T., Tjaden, J., Henrichs, G., Quick, W. P., Häusler, R., and Neuhaus, H. E. (1997). ADP-glucose drives starch synthesis in isolated maize endosperm amyloplasts: characterization of starch synthesis and transport properties across the amyloplast envelope. *Biochem. J.* 324, 503–509.

Münch, E. (1930). *Die Stoffbewegungen in der Pflanze*. Jena: Verlag Gustav Fischer, 234.

Nguyen-Quoc, B., and Foyer, C. H. (2001). A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. *J. Exp. Bot.* 52, 881–889. doi: 10.1093/jexbot/52.358.881

Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A. M., and Zeeman, S. C. (2004). A previously unknown maltose transporter essential for starch degradation in leaves. *Science* 303, 87–89. doi: 10.1126/science.1091811

Patrick, J. W., and Offler, C. E. (2001). Compartmentation of transport and transfer events in developing seeds. *J. Exp. Bot.* 52, 551–564. doi: 10.1093/jexbot/52.356.551

Paul, M. J., and Foyer, C. H. (2001). Sink regulation of photosynthesis. *J. Exp. Bot.* 52, 1383–1400. doi: 10.1093/jexbot/52.360.1383

Paul, M. J., and Pellny, T. K. (2003). Carbon metabolite feedback regulation of leaf photosynthesis and development. *J. Exp. Bot.* 54, 539–547. doi: 10.1093/jxb/erg052

Payyavula, R. S., Tay, K. H. C., Tsai, C.-J., and Harding, S. A. (2011). The sucrose transporter family in *Populus*: the importance of a tonoplast PtaSUT4 to biomass and carbon partitioning. *Plant J.* 65, 757–770. doi: 10.1111/j.1365-313X.2010.04463.x

Periappuram, C., Steinhauer, L., Barton, D. L., Taylor, D. C., Chatson, B., and Zou, J. (2000). The plastidic phosphoglucomutase from *Arabidopsis*. A reversible enzyme reaction with an important role in metabolic control. *Plant Physiol.* 122, 1193–1199. doi: 10.1104/pp.122.4.1193

Petreikov, M., Yeselson, L., Shen, S., Levin, I., Schaffer, A. A., Efrati, A., et al. (2009). Carbohydrate balance and accumulation during development of near-isogenic tomato lines differing in the AGPase-L1 allele. *J. Am. Soc. Hortic. Sci.* 134, 134–140.

Pommerrenig, B., Popko, J., Heilmann, M., Schulmeister, S., Dietel, K., Schmitt, B., et al. (2013). SUCROSE TRANSPORTER 5 supplies *Arabidopsis* embryos with biotin and affects triacylglycerol accumulation. *Plant J.* 73, 392–404. doi: 10.1111/tpj.12037

Poschet, G., Hannich, B., Raab, S., Jungkunz, I., Klemens, P. A. W., Krüger, S., et al. (2011). A novel *Arabidopsis* vacuolar glucose exporter is involved in cellular sugar homeostasis and affects the composition of seed storage compounds. *Plant Physiol.* 157, 1664–1676.

Rae, A. L., Jackson, M. A., Nguyen, C. H., and Bonnett, G. D. (2009). Functional specialization of vacuoles in sugarcane leaf and stem. *Tropical Plant Biol.* 2, 13–22. doi: 10.1007/s12042-008-9019-9

Rae, A. L., Perroux, J. M., and Grof, C. P. L. (2005). Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. *Planta* 220, 817–825. doi: 10.1007/s00425-004-1399-y

Ramaiah, M., Jain, A., Baldwin, J. C., Karthikeyan, A. S., and Raghothama, K. G. (2011). Characterization of the phosphate starvation-induced glycerol-3-phosphate permease gene family in *Arabidopsis*. *Plant Physiol.* 157, 279–291. doi: 10.1104/pp.111.178541

Rawsthorne, S. (2002). Carbon flux and fatty acid synthesis in plants. *Prog. Lipid Res.* 41, 182–196. doi: 10.1016/S0163-7827(01)00023-6

Reidel, E. J., Turgeon, R., and Cheng, L. (2008). A maltose transporter from apple is expressed in source and sink tissues and complements the *Arabidopsis* maltose export-defective mutant. *Plant Cell Physiol.* 49, 1607–1613. doi: 10.1093/pcp/pcn134

Reinders, A., Sivitz, A. B., Starker, C. G., Gantt, J. S., and Ward, J. M. (2008). Functional analysis of LjSUT4, a vacuolar sucrose transporter from *Lotus japonicus*. *Plant Mol. Biol.* 68, 289–299. doi: 10.1007/s11103-008-9370-0

Reinders, A., Sivitz, A. B., and Ward, J. M. (2012). Evolution of plant sucrose uptake transporters. *Front. Plant Sci.* 3:22. doi: 10.3389/fpls.2012.00022

Reiser, J., Linka, N., Lemke, L., Jeblick, W., and Neuhaus, H. E. (2004). Molecular physiological analysis of the two plastidic ATP/ADP transporters from *Arabidopsis*. *Plant Physiol.* 136, 3524–3536. doi: 10.1104/pp.104.049502

Renné, P., Dressen, U., Hebbeker, U., Hille, D., Flügge, U.-I., Westhoff, P., et al. (2003). The *Arabidopsis* mutant dct is deficient in the plastidic glutamate/malate translocator DiT2. *Plant J.* 35, 316–331.

Rennie, E. A., and Turgeon, R. (2009). A comprehensive picture of phloem loading strategies. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14162–14167. doi: 10.1073/pnas.0902279106

Riesmeier, J. W., Flügge, U.-I., Schulz, B., Heineke, D., Heldt, H.-W., Willmitzer, L., et al. (1993a). Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants. *Proc. Natl. Acad. Sci. U.S.A.* 90, 6160–6164.

Riesmeier, J. W., Hirner, B., and Frommer, W. B. (1993b). Potato sucrose transporter expression in minor veins indicates a role in phloem loading. *Plant Cell* 5, 1591–1598.

Riesmeier, J. W., Willmitzer, L., and Frommer, W. B. (1992). Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J.* 11, 4705–4713.

Riesmeier, J. W., Willmitzer, L., and Frommer, W. B. (1994). Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO J.* 13, 1–7.

Robinson, N. L., Hewitt, J. D., and Bennett, A. B. (1988). Sink metabolism in tomato fruit. I. Developmental changes in carbohydrate metabolizing enzymes. *Plant Physiol.* 87, 727–730. doi: 10.1104/pp.87.3.727

Rodríguez-Falcón, M., Bou, J., and Prat, S. (2006). Seasonal control of tuberization in potato: conserved elements with the flowering response. *Annu. Rev. Plant Biol.* 57, 151–180.

Rolletschek, H., Hosein, F., Miranda, M., Heim, U., Götz, K.-P., Schlereth, A., et al. (2005). Ectopic expression of an amino acid transporter (VfAAP1) in seeds of *Vicia narbonensis* and pea increases storage proteins. *Plant Physiol.* 137, 1236–1249.

Rolletschek, H., Nguyen, T. H., Häusler, R. E., Rutten, T., Göbel, C., Feussner, I., et al. (2007). Antisense inhibition of the plastidial glucose-6-phosphate/phosphate translocator in *Vicia* seeds shifts cellular differentiation and promotes protein storage. *Plant J.* 51, 468–484.

Rosche, E., Blackmore, D., Tegeder, M., Richardson, T., Schroeder, H., Higgins, T. J. V., et al. (2002). Seed-specific overexpression of a potato sucrose transporter increases sucrose uptake and growth rates of developing pea cotyledons. *Plant J.* 31, 165–175. doi: 10.1046/j.1365-313X.2002.01282.x

Ruan, Y.-L., and Patrick, J. W. (1995). The cellular pathway of postphloem sugar transport in developing tomato fruit. *Planta* 196, 434–444. doi: 10.1007/BF00203641

Ruan, Y.-L., Patrick, J. W., and Brady, C. (1997). Protoplast hexose carrier activity is a determinate of genotypic difference in hexose storage in tomato fruit. *Plant Cell Environ.* 20, 341–349. doi: 10.1046/j.1365-3040.1997.d01-73.x

Saftner, R. A., Daie, J., and Wyse, R. E. (1983). Sucrose uptake and compartmentation in sugar beet taproot tissue. *Plant Physiol.* 72, 1–6. doi: 10.1104/pp.72.1.1

Sauer, N. (2007). Molecular physiology of higher plant sucrose transporters. *FEBS Lett.* 581, 2309–2317. doi: 10.1016/j.febslet.2007.03.048

Sauer, N., and Stolz, J. (1994). SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. *Plant J.* 6, 67–77. doi: 10.1046/j.1365-313X.1994.6010067.x

Schaffer, A. A., and Petreikov, M. (1997). Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. *Plant Physiol.* 113, 739–746.

Schattat, M., Barton, K., Baudisch, B., Klösgen, R. B., and Mathur, J. (2011). Plastid stromule branching coincides with contiguous endoplasmic reticulum dynamics. *Plant Physiol.* 155, 1667–1677. doi: 10.1104/pp.110.170480

Schmitt, B., Stadler, R., and Sauer, N. (2008). Immunolocalization of solanaceous SUT1 proteins in companion cells and xylem parenchyma: new perspectives for phloem loading and transport. *Plant Physiol.* 148, 187–199. doi: 10.1104/pp.108.120410

Schmitz, J., Schöttler, M. A., Krüger, S., Geimer, S., Schneider, A., Kleine, T., et al. (2012). Defects in leaf carbohydrate metabolism compromise acclimation to high light and lead to a high chlorophyll fluorescence phenotype in *Arabidopsis thaliana*. *BMC Plant Biol.* 12:8. doi:10.1186/1471-2229-12-8

Schneider, A., Häusler, R. E., Kolukisaoglu, Ü., Kunze, R., van der Graaff, E., Schwacke, R., et al. (2002). An *Arabidopsis thaliana* knock-out of the chloroplast triose phosphate/phosphate transloctor is severely compromised only when starch synthesis, but not starch mobilization is abolished. *Plant J.* 32, 685–699.

Schneider, S., Hulpke, S., Schulz, A., Yaron, I., Höll, J., Imlau, A., et al. (2012). Vacuoles release sucrose via tonoplast-localised SUC4-type transporters. *Plant Biol.* 14, 325–336. doi: 10.1111/j.1438-8677.2011.00506.x

Schneidereit, A., Scholz-Starke, J., and Büttner, M. (2003). Functional characterization and expression analyses of the glucose-specific AtSTP9 monosaccharide transporter in pollen of *Arabidopsis*. *Plant Physiol.* 133, 182–190.

Schulz, A., Beyhl, D., Marten, I., Wormit, A., Neuhaus, E., Poschet, G., et al. (2011). Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2. *Plant J.* 68, 129–136. doi: 10.1111/j.1365-313X.2011.04672.x

Shannon, J. C., Pein, F. M., Cao, H. P., and Liu, K. C. (1998). Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into

amyloplasts of maize endosperm. *Plant Physiol.* 117, 1235–1252. doi: 10.1104/pp.117.4.1235

Shockey, J. M., Fulda, M. S., and Browse, J. A. (2002). *Arabidopsis* contains nine long-chain acyl-coenzyme A synthetase genes that participate in fatty acid and glycerolipid metabolism. *Plant Physiol.* 129, 1710–1722. doi: 10.1104/pp.003269

Sivitz, A. B., Reinders, A., Johnson, M. E., Krentz, A. D., Grof, C. P. L., Perroux, J. M., et al. (2007). *Arabidopsis* sucrose transporter SUC9. High-affinity transport activity, intragenic control of expression, and early flowering mutant phenotype. *Plant Physiol.* 143, 188–198. doi: 10.1104/pp.106.089003

Slewinski, T. L., Garg, A., Johal, G. S., and Braun, D. M. (2010). Maize SUT1 functions in phloem loading. *Plant Signal. Behav.* 5, 687–690. doi: 10.4161/psb.5.6.11575

Slewinski, T. L., Meeley, R., and Braun, D. M. (2009). Sucrose transporter1 functions in phloem loading in maize leaves. *J. Exp. Bot.* 60, 881–892. doi: 10.1093/jxb/ern335

Smidansky, E. D., Clancy, M., Meyer, F. D., Lanning, S. P., Blake, N. K., Talbert, L. E., et al. (2002). Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1724–1729. doi: 10.1073/pnas.022635299

Somerville, S. C., and Ogren, W. L. (1983). An *Arabidopsis thaliana* mutant defective in chloroplast dicarboxylate transport. *Proc. Natl. Acad. Sci. U.S.A.* 80, 1290–1294. doi: 10.1073/pnas.80.5.1290

Somerville, S. C., and Somerville, C. R. (1985). A mutant of *Arabidopsis* deficient in chloroplast dicarboxylate transport is missing an envelope protein. *Plant Sci. Lett.* 37, 217–220. doi: 10.1016/0304-4211(85)90007-0

Sonnewald, U. (2011). SWEETS – the missing sugar efflux carriers. *Front. Plant Sci.* 2:7. doi: 10.3389/fpls.2011.00007

Sonnewald, U., Brauer, M., von Schaewen, A., Stitt, M., and Willmitzer, L. (1991). Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. *Plant J.* 1, 95–106. doi: 10.1111/j.1365-313X.1991.00095.x

Sonnewald, U., Hajirezaei, M.-R., Kossmann, J., Heyer, A., Trethewey, R. N., and Willmitzer, L. (1997). Increased potato tuber size resulting from apoplastic expression of a yeast invertase. *Nat. Biotechnol.* 15, 794–797. doi: 10.1038/nbt0897-794

Srivastava, A. C., Ganesan, S., Ismail, I. O., and Ayre, B. G. (2008). Functional characterization of the *Arabidopsis* AtSUC2 sucrose/H+ symporter by tissue-specific complementation reveals an essential role in phloem loading but not in long-distance transport. *Plant Physiol.* 148, 200–211. doi: 10.1104/pp.108.124776

Stadler, R., Brandner, J., Schulz, A., Gahrtz, M., and Sauer, N. (1995). Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. *Plant Cell* 7, 1545–1554. doi: 10.1105/tpc.7.10.1545

Stadler, R., and Sauer, N. (1996). The *Arabidopsis thaliana* AtSUC2 gene is specifically expressed in companion cells. *Bot. Acta* 109, 299–306. doi: 10.1104/pp.008037

Stadler, R., Wright, K. M., Lauterbach, C., Amon, G., Gahrtz, M., Feuerstein, A., et al. (2005a). Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. *Plant J.* 41, 319–331. doi: 10.1111/j.1365-313X.2004.02298.x

Stadler, R., Lauterbach, C., and Sauer, N. (2005b). Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiol.* 139, 701–712. doi: 10.1104/pp.105.065607

Stettler, M., Eicke, S., Mettler, T., Messerli, G., Hörtensteiner, S., and Zeeman, S. (2009). Blocking the metabolism of starch breakdown products in *Arabidopsis* leaves triggers chloroplast degradation. *Mol. Plant* 2, 1233–1246. doi: 10.1093/mp/ssp093

Stitt, M. (1991). Rising CO2 levels and their potential significance for carbon flow in photosynthetic cells. *Plant Cell. Environ.* 14, 741–762. doi: 10.1111/j.1365-3040.1991.tb01440.x

Streatfield, S. J., Weber, A., Kinsman, E. A., Häusler, R. E., Li, J., Post-Beittenmiller, D., et al. (1999). The phosphoenolpyruvate/phosphate translocator is required for phenolic metabolism, palisade cell development, and plastid-dependent nuclear gene expression. *Plant Cell* 11, 1609–1621.

Sullivan, T. D., and Kaneko, Y. (1995). The maize brittle-1 gene encodes amyloplast membrane polypeptides. *Planta* 196, 477–484. doi: 10.1007/BF00203647

Tegeder, M., Wang, X.-D., Frommer, W. B., Offler, C. E., and Patrick, J. W. (1999). Sucrose transport into developing seed of *Pisum sativum* L. *Plant J.* 18, 151–161. doi: 10.1046/j.1365-313X.1999.00439.x

Thorbjörnsen, T., Villand, P., Denyer, K., Olsen, O.-A., and Smith, A. M. (1996). Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J.* 10, 243–250. doi: 10.1046/j.1365-313X.1996.10020243.x

Tjaden, J., Möhlmann, T., Kampfenkel, K., Henrichs, G., and Neuhaus, H. E. (1998). Altered plastidic ATP/ADP-translocator activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *Plant J.* 16, 531–540. doi: 10.1046/j.1365-313x.1998.00317.x

Trentmann, O., Jung, B., Neuhaus, H. E., and Haferkamp, I. (2008). Non-mitochondrial ATP/ADP transporters accept phosphate as third substrate. *J. Biol. Chem.* 283, 36486–36493. doi: 10.1074/jbc.M806903200

Truernit, E., and Sauer, N. (1995). The promoter of the *Arabidopsis thaliana* sucrose-H+ symporter gene directs expression of β-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. *Planta* 196, 564–570. doi: 10.1007/BF00203657

Turgeon, R. (1989). The sink-source transition in leaves. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40, 119–138. doi: 10.1146/annurev.pp.40.060189.001003

Uys, L., Botha, F. C., Hofmeyr, J.-H. S., and Rohwer, J. M. (2007). Kinetic model of sucrose accumulation in maturing sugarcane culm tissue. *Phytochemistry* 68, 2375–2392. doi: 10.1016/j.phytochem.2007.04.023

van Dongen, J. T., Ammerlaan, A. M. H., Wouterlood, M., van Aelst, A. C., and Borstlap, A. C. (2003). Structure of the developing pea seed coat and the post-phloem transport pathway of nutrients. *Ann. Bot.* 91, 729–737. doi: 10.1093/aob/mcg066

Vigeolas, H., Möhlmann, T., Martini, N., Neuhaus, H. E., and Geigenberger, P. (2004). Embryo-specific reduction of ADP-Glc pyrophosphorylase leads to an inhibition of starch synthesis and a delay in oil accumulation in developing seeds of oilseed rape. *Plant Physiol.* 136, 2676–2686. doi: 10.1104/pp.104.046854

Viola, R., Roberts, A. G., Haupt, S., Gazzani, S., Hancock, R. D., Marmiroli, N., et al. (2001). Tuberization in potato involves a switch from apoplastic to symplastic phloem unloading. *Plant Cell* 13, 385–398.

Vitale, A., and Ceriotti, A. (2004). Protein quality control mechanisms and protein storage in the endoplasmic reticulum. A conflict of interests? *Plant Physiol.* 136, 3420–3426. doi: 10.1104/pp.104.050351

Voll, L., Häusler, R. E., Hecker, R., Weber, A., Weissenböck, G., Fiene, G., et al. (2003). The phenotype of the *Arabidopsis* cue1 mutant is not simply caused by a general restriction of the shikimate pathway. *Plant J.* 36, 301–317.

von Schaewen, A., Stitt, M., Schmidt, R., Sonnewald, U., and Willmitzer, L. (1990). Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. *EMBO J.* 9, 3033–3044.

Walters, R. G., Ibrahim, D. G., Horton, P., and Kruger, N. J. (2004). A mutant of *Arabidopsis* lacking the triose-phosphate/phosphate translocator reveals metabolic regulation of starch breakdown in the light. *Plant Physiol.* 135, 891–906. doi: 10.1104/pp.104.040469

Wang, Z., Chen, X., Wang, J., Liu, T., Liu, Y., Zhao, L., et al. (2007). Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. *Plant Cell Tissue Organ Cult.* 88, 83–92. doi: 10.1007/s11240-006-9173-4

Weber, A., Servaites, J. C., Geiger, D. R., Kofler, H., Hille, D., Gröner, F., et al. (2000). Identification, purification, and molecular cloning of a putative plastidic glucose translocator. *Plant Cell* 12, 787–802.

Weber, H., Borisjuk, L., Heim, U., Sauer, N., and Wobus, U. (1997). A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. *Plant Cell* 9, 895–908. doi: 10.1105/tpc.9.6.895

Weise, A., Barker, L., Kühn, C., Lalonde, S., Buschmann, H., Frommer, W. B., et al. (2000). A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of

plants. *Plant Cell* 12, 1345–1355. doi: 10.1105/tpc.12.8.1345

Weise, S. E., Weber, A. P. M., and Sharkey, T. D. (2004). Maltose is the major form of carbon exported from the chloroplast at night. *Planta* 218, 474–482. doi: 10.1007/s00425-003-1128-y

Werner, D., Gerlitz, N., and Stadler, R. (2011). A dual switch in phloem unloading during ovule development in *Arabidopsis*. *Protoplasma* 248, 225–235. doi: 10.1007/s00709-010-0223-8

Weschke, W., Panitz, R., Sauer, N., Wang, Q., Neubohn, B., Weber, H., et al. (2000). Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *Plant J.* 21, 455–467. doi: 10.1046/j.1365-313x.2000.00695.x

Whittaker, A., and Botha, F. C. (1997). Carbon partitioning during sucrose accumulation in sugarcane intermodal tissue. *Plant Physiol.* 115, 1651–1659. doi: 10.1104/pp.115.4.1651

Wingenter, K., Schulz, A., Wormit, A., Wic, S., Trentmann, O., Hoermiller, I. I., et al. (2010). Increased activity of the vacuolar monosaccharide transporter TMT1 alters cellular sugar partitioning, sugar signaling, and seed yield in *Arabidopsis*. *Plant Physiol.* 154, 665–677. doi: 10.1104/pp.110.162040

Wingenter, K., Trentmann, O., Winschuh, I., Hörmiller, I. I., Heyer, A. G., Reinders, J., et al. (2011). A member of the mitogen-activated protein 3-kinase family is involved in the regulation of plant vacuolar glucose uptake. *Plant J.* 68, 890–900. doi: 10.1111/j.1365-313X.2011.04739.x

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., and Provart, N. J. (2007). An 'electronic fluorescent pictograph' browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* 2:e718. doi: 10.1371/journal.pone.0000718

Wippel, K., and Sauer, N. (2012). *Arabidopsis* SUC1 loads the phloem in suc2 mutants when expressed from the SUC2 promoter. *J. Exp. Bot.* 63, 669–679. doi: 10.1093/jxb/err255

Wormit, A., Trentmann, O., Feifer, I., Lohr, C., Tjaden, J., Meyer, S., et al. (2006). Molecular identification and physiological characterization of a novel monosaccharide transporter from *Arabidopsis* involved in vacuolar sugar transport. *Plant Cell* 18, 3476–3490. doi: 10.1105/tpc.106.047290

Wyse, R. (1979). Sucrose uptake by sugar beet tap root tissue. *Plant Physiol.* 64, 837–841. doi: 10.1104/pp.64.5.837

Yamada, K., Osakabe, Y., Mizoi, J., Nakashima, K., Fujita, Y., Shinozaki, K., et al. (2010). Functional analysis of an *Arabidopsis thaliana* abiotic-stress-inducible facilitated diffusion transporter for monosaccharides. *J. Biol. Chem.* 285, 1138–1146. doi: 10.1074/jbc.M109.054288

Yu, T.-S., Kofler, H., Häusler, R. E., Hille, D., Flügge, U.-I., Zeeman, S. C., et al. (2001). The *Arabidopsis sex1* mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell* 13, 1907–1918.

Zhang, L., Häusler, R. E., Greiten, C., Hajirezaei, M.-R., Haferkamp, I., Neuhaus, H. E., et al. (2008). Overriding the co-limiting import of carbon and energy into tuber amyloplasts increases the starch content and yield of transgenic potato plants. *Plant Biotechnol. J.* 6, 453–464. doi: 10.1111/j.1467-7652.2008.00332.x

Zhang, X.-Y., Wang, X.-L., Wang, X.-F., Xia, G.-H., Pan, Q.-H., Fan, R.-C., et al. (2006). A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. *Plant Physiol.* 142, 220–232. doi: 10.1104/pp.106.081430

Zhou, Y., Chan, K., Wang, T. L., Hedley, C. L., Offler, C. E., and Patrick, J. W. (2009). Intracellular sucrose communicates metabolic demand to sucrose transporters in developing pea cotyledons. *J. Exp. Bot.* 60, 71–85. doi: 10.1093/jxb/ern254

Zhou, Y., Qu, H., Dibley, K. E., Offler, C. E., and Patrick, J. W. (2007). A suite of sucrose transporters expressed in coats of developing legume seeds includes novel pH-independent facilitators. *Plant J.* 49, 750–764. doi: 10.1111/j.1365-313X.2006.03000.x

Zhu, Y. J., Komor, E., and Moore, P. H. (1997). Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiol.* 115, 609–616.

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Using evolution as a guide to engineer Kranz-type C_4 photosynthesis

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Kranz-type C_4 photosynthesis has independently and rapidly evolved over 60 times to dramatically increase radiation use efficiency in both monocots and eudicots. Indeed, it is one of the most exceptional examples of convergent evolution in the history of life. The repeated and rapid evolution of Kranz-type C_4 suggests that it may be a derivative of a conserved developmental pathway that is present in all angiosperms. Here, I argue that the Kranz-type C_4 photosynthetic system is an extension of the endodermis/starch sheath, that is normally only found in the roots and stems, into photosynthetic structures such as leaves. Support for this hypothesis was recently provided by a study that showed that the same genetic pathway that gives rise to the endodermis in roots, the SCARECROW/SHORT-ROOT radial patterning system, also regulates the development of Kranz anatomy and C_4 physiology in leaves. This new hypothesis for the evolution of Kranz-type C_4 photosynthesis has opened new opportunities to explore the underlying genetic networks that regulate the development and physiology of C_4 and provides new potential avenues for the engineering of the mechanism into C_3 crops.

Keywords: Kranz anatomy, C_4 photosynthesis, bundle sheath, endodermis, SCARECROW, SHORT-ROOT, phyllode theory, evolution

THEORY AND DISCUSSION

A new revolution in agriculture is needed to keep pace with the demands of humanity in the next century (Fedoroff et al., 2010). More humans will be alive at one time than ever before in earth's history. Human population has been on the same trajectory for decades, regardless of food availability (Fedoroff and Cohen, 1999). Until recently, food production was able to stay ahead of the overall needs of the population. The first green revolution provided enough food to avoid mass starvation at the time of its implementation, as well as a surplus to cope with the population increase in the last half century (Borlaug, 2007). But now, based on our current resource availability, agricultural productivity, and projected consumption rates, some suggest we will approach or surpass human carrying capacity on the planet (Borlaug, 2002). The gap between agricultural surplus and human needs is narrowing fast, or has at this point in time, closed.

Crop breeding and biotechnology in the last century have altered plants in many drastic ways. Breeders were able to increase the harvest index, growth rate, biomass accumulation, disease and pest resistance, biotic and abiotic stress tolerance, and nutrient use efficiency, while also extending the climatic range of crops into previously unproductive regions (Fischer and Edmeades, 2010). However, the rate of improvement in these areas is still outpaced by human demands (Brown, 2012). Increases in actualized yields are becoming harder to achieve (Reynolds et al., 2011). Additionally, most beneficial traits that have been exploited to date also require a simultaneous increase in inputs.

One trait that has not significantly changed is the efficiency of photosynthesis (Reynolds et al., 2011). The maximum productive output per unit photosynthetic area, i.e., radiation use efficiency (RUE), has remained steady throughout domestication and selective breeding of most crops (Reynolds et al., 2009). If this previously recalcitrant trait could be modified, it could open up a new avenue of crop improvement. Now with the use of biotechnology, it has been suggested that increasing the RUE in C_3 crops, by introducing the C_4 photosynthetic mechanism, could be an effective way to increase yields by boosting productivity per unit area of land as well as reducing the amount of water and nitrogen used in achieving those yields (Hibberd et al., 2008; von Caemmerer et al., 2012). Integrating C_4 photosynthesis into C_3 crops may become even more necessary if climatic changes continue along the current trends (Sage and Zhu, 2011). For example, a recent report of soybean production in the Midwest of the USA revealed that the predicted fertilization effect of increased CO_2 in the future may be negated by the increasing temperatures predicted for the coming decades (Ruiz-Vera et al., 2013). Thus, the one positive aspect of increased anthropogenic CO_2 emissions that has been argued by many scientists may be undermined by the broader impacts of climate change.

Kranz-type C_4 photosynthesis is one mechanism that plants have repeatedly and rapidly evolved to dramatically increase RUE and stress tolerance in hot and dry environments by reducing the rate of photorespiration in the carbon fixation process (for reviews and discussion on the biochemistry of C_4 photosynthesis, see Langdale, 2011; Wang et al., 2011). This adaption has become even more effective in recent planetary history when carbon dioxide levels declined and oxygen levels increased. Current conditions are a stark contrast to the environment in which photosynthesis and Rubisco, the enzyme that fixes CO_2 for entry into the Calvin cycle, first evolved (Sage, 2004). Thus it can be argued that, the

more ancient C_3 mechanism is best adapted for an environment that no longer exists. In contrast, the C_4 mechanism overcomes the inherent limitations of Rubisco by dividing the photosynthetic process into two cell types. These cells are arranged into concentric circles around veins that produce a wreath-like appearance known as Kranz anatomy (Langdale et al., 1989). The bundle sheath (BS) cells comprise the inner circle attached to the vein and are responsible for the key reductive step in photosynthesis, carried out by Rubisco (Sage and Zhu, 2011; Sage et al., 2012; von Caemmerer et al., 2012). The mesophyll (M) cells encircle the BS and are responsible for the initial CO_2 fixation by phosphoenolpyruvate carboxylase to produce the 4-C compounds malate or aspartate (Furbank, 2011). Malate or aspartate then moves from the M to the BS cells through plasmodesmata where CO_2 is then released and then re-fixed by Rubisco. This process concentrates CO_2 around Rubisco while also excluding oxygen in the BS, thus eliminated the metabolic drag of photorespiration that is common in C_3 photosynthesis (Sage et al., 2012).

Previously, it was proposed that there are five major phases of morphological and physiological adaptations that plants undergo in the evolutionary trajectory toward C_4 photosynthesis (Sage et al., 2012). The first proposed step is preconditioning which includes increasing vein density and possible gene duplication. Second is modification of BS cells. This includes cell enlargement, production of more organelles, and altered localization of the chloroplasts and mitochondria. M cell volume is also reduced during this transition. Together these changes lead to a "Proto-Kranz" condition. Third is the installation of the basic photorespiratory CO_2 pump which includes the reduction of the M:BS cell ratio, localization of the C_3 cycle to the BS and activation of the basic C_2 system. Fourth is the enhancement of C_4 CO_2 metabolic capture and pump cycle within the M cells, which includes up-regulation and M-specific expression of phosphoenolpyruvate carboxylase. Finally, in the optimization phase, anatomy and biochemistry are fine tuned to exploit the full efficiency of the C_4 mechanism (Sage, 2004).

However, there are reasons to suggest that the evolutionary progression toward the C_4 state has been rapid. Kranz-type C_4 has independently evolved over 60 times, occurring throughout the angiosperms in both monocots and eudicots (Sage et al., 2011). Indeed, it is one of the most exceptional examples of convergent evolution in the history of life. Astonishingly, Kranz-type C_4 appears almost "fully formed" in each of the evolutionary events where it has arisen (Langdale, 2011). There is little evidence that is a slow evolutionary progression toward the C_4 state as C_3–C_4 intermediates are lacking for most of the extant C_4 species. Many C_4 species also have closely related C_3 relatives suggesting recent and rapid appearance of the C_4 syndrome within some families of plants (Sage et al., 2011). Indeed, Kranz-type C_4 is a classic example of Goldschmidt's "Hopeful Monsters" (Goldschmidt, 1933), in which spontaneous complexity rapidly appears in some branches of life, and cannot be easily explained in the Darwinian model of evolution. However, I interpret the repeated and rapid evolution of complete Kranz-type C_4 differently. This "fully formed" phenomenon (Langdale, 2011) suggests that only simple changes in some of the innate genetic programs are required in order for C_4 to arise from a C_3 background (Westhoff and Gowik, 2010). Therefore, it is reasonable to hypothesize that Kranz-type C_4 may be a modification or extension of a conserved morphogenetic pathway that is inherent to all of the angiosperms.

What conserved tissue or genetic program in C_3 plants could give rise to such a complex mechanism as Kranz-type C_4 photosynthesis? If we take the view that cells such as the BS are derived from other cells that are already programmed with many of the underlying C_4 biochemical programs, it is reasonable to hypothesize that the BS cells themselves confer the underlying properties of the C_4 mechanism. The reasoning behind this hypothesis is that all living cells are programed with a specific "identity" that is determined at the time when ground meristem cells initiate differentiation. For example, cells that create the boundary between the outside environment and the internal organs all have a shared epidermis "identity." They may have various characteristics depending on their location on the plant and specific function, but all share similar morphological and physiological properties as well as underlying developmental and genetic programs. Thus, root epidermal cells share identity with leaf or stem epidermal cells. Therefore, it is possible that C_4 BS cells may share a similar identity with cells elsewhere in the plant. In the case of Kranz-type C_4 cells, this shared identity may confer the underlying programs needed to establish or precondition the C_4 metabolic mechanism within in the context of photosynthetic tissues (Slewinski et al., 2012).

What other cells within all angiosperms could be similar to C_4 BS cells? Katherine Esau may have already answered this question when she published her anatomical surveys in the 1940s and 1950s – before C_4 was discovered (Esau, 1953). She described some atypical species of plants that had "starch sheaths" within the photosynthetic leaf blades. She also described all BS tissue in leaves as having properties of endodermal tissue. When we look back on these observations we find something striking. Many of the atypical plants that Esau (1953) described as having "starch sheaths" in the photosynthetic leaf blades, turned out to be Kranz-type C_4 plants such as maize and sorghum. Indeed, we now know that the Kranz C_4 BS in leaves share similarities with endodermal tissues in petioles, stems, and roots (Nelson and Dengler, 1997; Slewinski et al., 2012). In all of these tissues, the endodermis is comprised of a single cell layer that surrounds the vasculature, has suberized cell walls, and displays polar expression of the pin-formed (PIN) effluxors, which conduct auxin through this cell layer (Slewinski et al., 2012). Based on Esau's detailed observations of leaf anatomy and a plethora of recent reports on C_4 physiology and development, I present a new hypothesis for the rapid and repeated evolution of C_4 photosynthesis in the angiosperms.

HYPOTHESIS

The Kranz-type C_4 photosynthetic mechanism arises when the endodermal/starch sheath program extends into photosynthetic structures, such as leaves, where it is normally repressed or underdeveloped. This leads to a synergistic interaction which can produce the novel C_4 pathway from underlying components of both the C_3 photosynthetic program and anatomical and metabolic features of the endodermis/starch sheath.

In other words, this suggests that the Kranz-type C_4 mechanism is the context-specific manifestation of the endodermis in a

photosynthetic tissue. The C_4 condition arises when the endodermis projects into the photosynthetic tissues, which also extends the properties of the endodermal/starch sheath program from stem and petiole into the leaf (Slewinski et al., 2012). A schematic of this hypothesis is presented in **Figure 1**. Thus, the inherent physiology of the endodermis may integrate into the photosynthetic program, resulting in a new synergistic physiology, which we know as C_4 photosynthesis.

In plants, the tissue in which a cell resides usually determines the cell's function and physiological properties. This reasoning can be applied to the endodermis, which is a dynamic tissue that appears to have context-dependent functions. For example, in roots the endodermis encircles the vascular core of the root and acts as an internal barrier for solute transport from the cortex and epidermal cell layers that interact with the external soil environment (Alassimone et al., 2012). At the root tip, columella cells have different properties than their adjacent stem cells, which are also part of the endodermal tissue (Welch et al., 2007; Ogasawara et al., 2011). Along the root length, endodermal cells do not accumulate starch whereas the endodermis in the stem and petiole does, and is thus termed the "starch sheath" (Wysocka-Diller et al., 2000). The starch sheath usually extends along the vascular core(s) from the base of the shoot–root junction to the petiole–leaf blade junction. The starch-filled amyloplasts within these cells act as statoliths – providing gravity cues to the cells in a similar manner to the columella cells within the root tip (Morita et al., 2007). Within these cells, the amyloplasts display a polar localization at the base of the cell, in the direction of gravitational pull. Changes in amyloplast position in these cells trigger changes in auxin transport through the endodermal cell layer (Tanimoto et al., 2008). This results in differential cell expansion in the stem that properly orients the plant into the upright position, opposing the direction of gravity (Morita et al., 2007).

When comparing the many forms of endodermis that occur in plants, the C_4 BS is most similar to that of the starch sheath in petioles and stems (Hibberd and Quick, 2002; Tanimoto et al., 2008). Interestingly, C_4 chloroplasts are also similar to those found in the starch sheath of the stem and petiole in certain ways. First, the C_4 BS chloroplasts preferentially accumulate starch when compared to M chloroplasts (Lunn and Furbank, 1997). Second, C_4 chloroplasts usually have a fixed location in the cells, either on the cell surface adjacent to the vascular core or adjacent to the M (Morita et al., 2007). Third, in many C_4 species, BS chloroplasts lack photosystem II and stacked thylakoid grana, similar to amyloplasts found in the starch sheath (Langdale, 2011). Although, there is great variation in all three of these characteristics in C_4 species, similarities suggest that chloroplasts within the starch sheath and the C_4 BS share components of their identity. Is it possible that chloroplasts in the C_4 BS are essentially photosynthetic-amyloplasts, i.e., plastids of hybrid identity? This may explain why dimorphic chloroplasts are frequently associated with the C_4 BS cells, because the BS cells have a mixed identity of both the starch sheath and photosynthetic cells. However, there is wide variation in BS chloroplast structure within the Kranz-type and single celled C_4 species, suggesting that a range of amyloplast-like features are compatible with C_4 BS, and that only a subset of associated starch sheath/amyloplasts mechanisms are required or sufficient to produce a functional C_4 photosynthetic system.

In an insightful paper by Hibberd and Quick (2002), it was shown that the starch sheath in aerial parts of the plant, especially petioles, is involved in internal CO_2 recycling (Hibberd and Quick, 2002). Respiring tissues such as roots produce abundant CO_2 as a waste product. However, not all of the CO_2 is released into the soil environment that surrounds the roots (Bloemen et al., 2013). Much of the respired carbon migrates into the xylem stream that flows from the roots toward the leaves. A study using mature poplar trees shows that a significant portion of the

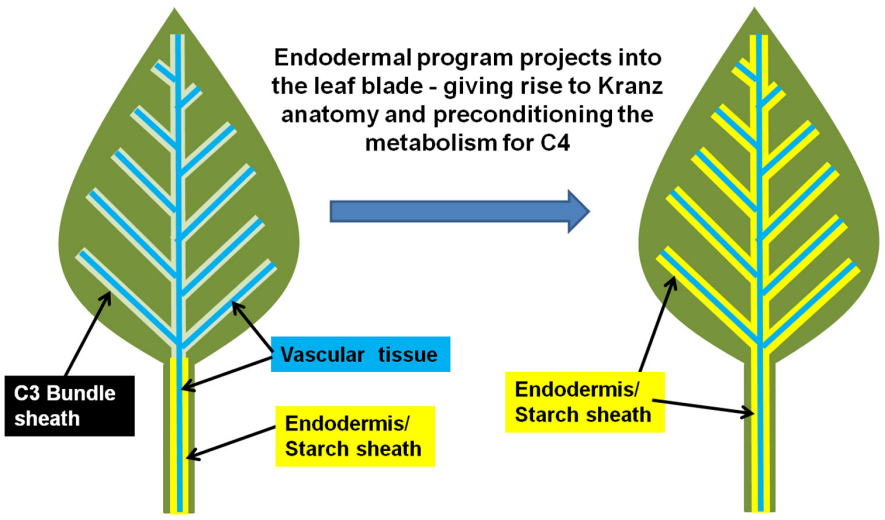

FIGURE 1 | Model for the evolution of Kranz-type C_4 in dicots. A normal C_3 dicot leaf is represented on the right. The endodermis/starch sheath (yellow) is present on the vasculature (blue) of the petiole and lower leaf zone, but is absent in the leaf blade/upper leaf zone. The hypothesized shift from C_3 to Kranz-type C_4 may arise when the endodermal/starch sheath program extends out from the petiole/lower leaf zone into the leaf blade.

respired carbon in roots eventually ends up re-fixed in the petioles at the base of leaves (Bloemen et al., 2013). This carbon is most likely in the form of malate (Hibberd and Quick, 2002), a neutral compound that does not impact pH like carbonic acid, which is produced when CO_2 dissolves either in the cytosol or apoplastic water reserves which flow into the xylem stream. In *Arabidopsis*, tobacco and celery xylem-derived malate is re-assimilated in the photosynthetic endodermal/starch sheath cells that surround the vasculature within the petiole and leaf mid-vein (Hibberd and Quick, 2002). Most of this carbon ends up in starch during the day, then is mobilized and transported in the phloem in the form of sucrose to sink tissues at night. It is reasonable to hypothesize that this is the precursor mechanism that gives rise to CO_2 metabolic shuffling in the C_4 mechanism. In other words, C_4 metabolic shuffling may be an extension of the internal CO_2 recycling system. In the case of Kranz-type C_4, this CO_2 waste management system extends out from the petiole with the endodermal program, giving rise to both Kranz anatomy while also preconditioning the leaf tissue for intercellular CO_2 metabolic shuffling. It is likely that once the full endodermal/starch sheath program extends into the leaf, the synergistic interaction between the photosynthetic cells and the endodermis initiates the C_4 metabolic mechanism, schematically represented in **Figure 2**. The initial event may not generate a fully functional C_4 mechanism immediately, but may give rise to the so called "C_3–C_4 intermediates" which possess the correct architecture, and have properties of both the C_3 and C_4 mechanisms. Further selection for the C_4 mechanism may be required to suppress the remnants of the C_3 photosynthetic pathway that are unneeded or redundant, while concurrently enhancing the more dominant features of the C_4 metabolic pathway. This is not to say that C_3–C_4 intermediates always represent a transitional stage; they may be fully adapted in their current form in many cases (Sage et al., 2011).

It can be argued that selection against the C_4/starch sheath physiological program in the C_3–C_4 intermediates is just as likely (Vicentini et al., 2008; Langdale, 2011). A full reversal of C_4 to C_3 is also possible and has already been reported in some of the C_3 grasses (Vicentini et al., 2008). As a result, plants could arise that possess Kranz/C_4-like anatomical features but with C_3 photosynthetic metabolism. Another possibility is that C_3–C_4 intermediates, arising from either selection for or against the Kranz-type C_4 pathway, could have some of the advantageous characteristics of full C_4 plants in hot and dry environments (Sage et al., 2011). Thus, it can also be argued that development of C_4-like traits can confer fitness on their own, implying that the C_3–C_4 intermediate state is an independent evolutionary trajectory (Langdale, 2011; Sage et al., 2011). Overall, this new view of C_4 evolution suggests that only small changes are required to rapidly produce dramatic diversity in anatomy and physiology. This diversity is then subject to selection for or against the C_4 mechanism based on the environmental pressures of the organism.

Selection for enzymatic cell specificity may also be necessary to increase the CO_2 metabolic pump from the M to the BS, while also concurrently enriching Rubisco in the BS. Presumably, these two processes would evolve in parallel because sequestration of Rubisco to the BS without the CO_2 pump would reduce carbon fixation in free air and lead to an evolutionary

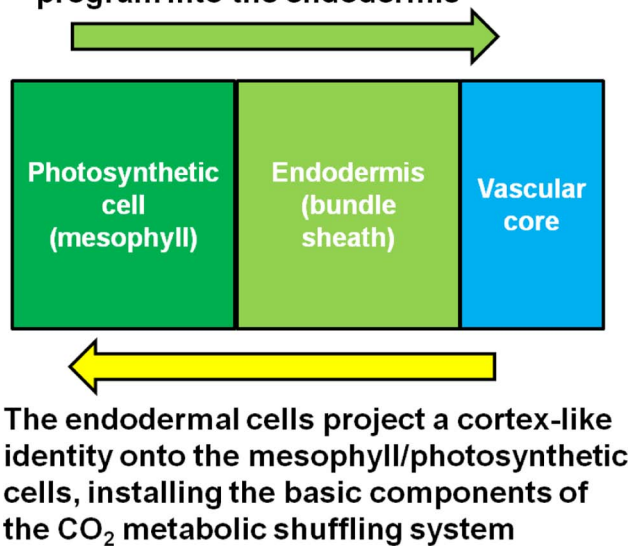

FIGURE 2 | Model for the synergistic interaction between the starch sheath and the photosynthetic cells. Kranz-type C_4 may arise when the photosynthetic system of the leaf integrates with the CO_2 metabolic shuffling system usually found in the respiring tissues of the plant.

disadvantage. Interestingly, only phosphoenolpyruvate carboxylase is common to all of the decarboxylation types of C_4 (Sage, 2004; Furbank, 2011). Extrapolation of the underlying endodermal physiology may occur differently with each independent evolutionary event – leading to variations in the CO_2 metabolic pump, i.e., Nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME), Nicotinamide adenine dinucleotide-malic enzyme (NAD-ME), or phosphoenolpyruvate carboxykinase (PEPCK) types (Sage et al., 2011). However, recent evidence suggests that these three decarboxylation types may not be distinct, but are flexible depending on environmental and developmental conditions (Furbank, 2011; Pick et al., 2011). Within the grasses, switching of decarboxylation types within a species has been reported (Vicentini et al., 2008). However, if the three decarboxylation types are extrapolations of the underlying physiology of the endodermis/starch sheath program, then it is reasonable to hypothesize that each type is simply a dominate enzymatic pathway within a larger physiological context that includes subtle forms of the other two types. Section pressures on a recently evolved C_4 species then determines which of the three decarboxylation types become dominant. The other pathways are most likely not eliminated in this selection but left in their original and more subtle "housekeeping" roles or suppressed to lower levels. Thus under this new hypothesis, significant plasticity and flexibility in the C_4 mechanism would also be conferred by the underlying endodermal/starch sheath program.

Following these arguments, it is important to also highlight that, in both roots and stems, the endodermis functions as a high-capacity auxin conducting tissue (Alassimone et al., 2012). In both C_3 and C_4 plants, vein patterning is regulated by auxin gradients

generated by both synthesis and transport (Scarpella et al., 2010). Auxin produced in the epidermis drains toward preexisting veins within the developing tissues. When larger veins form, they also produce auxin gradients within the adjacent ground meristem tissue by depleting auxin from the surrounding cells. This creates an auxin minima that initiates the formation of smaller vein orders that form after the larger orders of veins have been established and are undergoing differentiation (Scarpella et al., 2010; Gardiner et al., 2011). The formation pattern of minor veins between established major and intermediate veins in maize is shown in **Figure 3**. The extension of the endodermal layer into the vascular tissue in the developing leaf may enhance the depletion of auxin from the ground tissue in C_4 leaves when compared to developing C_3 leaves. Under these assumptions, it is reasonable to hypothesize that the increased vein density observed in C_4 plants is, at least in part, due to the increased auxin depletion associated with the developing endodermal layer. This would presumably create more or stronger auxin minima, thus initiating more minor veins.

Unlike non-Kranz species, each vein initiation confers the formation of entire Kranz units (vascular core, BS, and surrounding M cells; Nelson and Dengler, 1997). It is unlikely that veins can get closer than one vascular Kranz unit because of the nature of the underlying endodermal developmental program. In this model, the development and identity of the cells is determined by the signals generated from the vascular core – which first determines BS. BS cells then generate signals that determine M specification. Thus, the proximity and intercellular interactions from the endodermal developmental program may also confer a cortex-like identity on the already present photosynthetic M cells, modifying their development, architecture, and physiology. This may explain why C_4 plants reduce M cell counts to the extent that they match BS cells, ultimately ending in a 1:1 ratio. In contrast, veins in C_3 plants do not form such units. Rather they form in a pool of ground meristem cells – defining cells that will become part of the vasculature and excluding the cells that will give rise to the M. Thus, the C_3 mode of vascular development leads to more variable numbers of M cells between vascular strands.

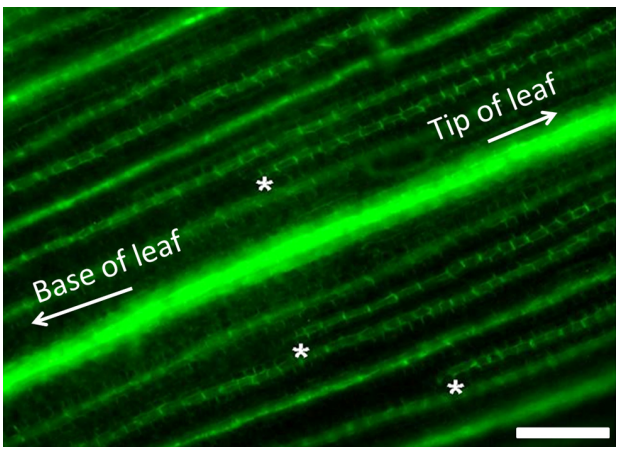

FIGURE 3 | Minor vein formation in developing maize leaves. Visualization of PIN-YFP vascular marker in developing maize leaves showing minor vein formation. Minor veins initiate at the tip of the leaf and develop toward the base between the established large lateral and intermediate veins. Developing tips of minor veins are demarcated with white asterisks. Scale bar: 50 μm.

The shift in plasmodesmata density and specialization at the M–BS interface in the leaves of C_4 plants may also be a pleiotropic effect of the endodermal program in the leaf. In other parts of the plant, the endodermis is coated with suberin and other hydrophobic compounds that create an apoplastic barrier that limits cell-to-cell flow of water and solutes through the cell wall (Geldner, 2013). Therefore most transport between the endodermal cells and the surrounding cortex or parenchyma cells is restricted to the symplastic route – through abundant plasmodesmata that connect the cytosolic domains of adjacent cells. Although the suberized apoplastic barrier is only sometimes associated with the C_4 BS (Sage, 2004), increased intercellular symplastic transport between BS and M cells appears to be necessary for an efficient CO_2 metabolic pump between M and BS cells. As with the other traits associated with C_4 specialization mentioned above, the intercellular transport mechanism utilized by the C_4 BS and M cells in the leaf may be an extension and modification of the system found in the endodermal tissue in the roots and stems.

In *Arabidopsis* the genes that underpin endodermis formation, *Scarecrow* (SCR) and *Short-root* (SHR), are expressed in roots, stems, and leaves (Wysocka-Diller et al., 2000; Gardiner et al., 2011). The *SHR* gene is expressed in cells within the vascular core (Helariutta et al., 2000), except for the phloem initial cells (Yu et al., 2010). The SHR protein moves out from the vascular core cells and activates the *Scr* gene within the cells that are in contact with the vascular core (Koizumi et al., 2012). SCR protein binds to SHR and sequesters the protein in the nucleus, preventing further movement (Wu and Gallagher, 2012). This mechanism deliminates a single cell layer as well as initiates the cascade of signals that establish endodermis identity. Thus, it is reasonable to hypothesize that if Kranz-type BS tissue is just an extension of the endodermal program, they should also be subject to mutations in the essential endodermal patterning and development genes SCR and SHR. Indeed, support for this reasoning was recently provided. It was shown that the maize ortholog of SCR plays a role in BS development in maize leaves (Slewinski et al., 2012). Mutations in the *ZmSCR* gene result in proliferation of BS cells, altered differentiation of BS chloroplasts, vein distortion, and reduction in minor vein formation and overall vein density. *zmscr* mutant plants also produce starch-less BS cells that closely resemble starch-less stem endodermal cells in the *shr* mutant of *Arabidopsis* called *endodermal amyloplasts less1* or *eal1* (Morita et al., 2007). In the *scr* mutant of maize, some of these starch-less cells also have altered plasmodesmata within the cell walls that separate the BS and M cells (Slewinski et al., 2012), suggesting that their specialization is also linked to the endodermal program. Thus, this provides for the first time, genetic evidence that the endodermal development pathway underlies C_4 BS development. This study also suggests, though does not directly prove, that SHR also plays a critical role in the development of the BS and underlying metabolism in C_4 plants.

Analysis of the *large scutellar node* (*lsn*) mutant of maize also supports the endodermal development model for C_4 BS in leaves. The *lsn* mutant phenotype mimics the abnormalities

observed when auxin transport inhibitors are applied to developing leaves (Landoni et al., 2000). These abnormalities include vein distortions, vascular hypertrophy, and disorganized vascular core structure (**Figures 4A,B**). What is interesting in the *lsn* mutant is the formation of normal BS and M, both structurally and physiologically, around the distorted vascular core in the leaves (**Figures 4C,D**; Landoni et al., 2000). *lsn* BS cells preferentially accumulate starch like wild type plants (**Figures 4E,F**), and both BS and M plastids appear normal in transmission electron microscopy (TEM) analysis (**Figures 4G,H**). This finding conflicts with the cell lineage models that have previously been proposed for the development of the C_4 BS which suggested that BS and M cells arose from organized cell division patterns (Langdale et al., 1989; Sud and Dengler, 2000). However, BS formation in *lsn* more closely resembles the endodermis that surrounds distorted veins in *Arabidopsis* plants grown in the presence of auxin transport inhibitors (Wysocka-Diller et al., 2000), suggesting that organized and coordinated cell division is not essential for the development of Kranz anatomy. Although, analysis of *lsn* does fit within the framework of the endodermis/starch sheath developmental model (Helariutta et al., 2000). Additionally in *Arabidopsis*, the SCR::GFP construct is still only expressed in a single cell layer of endodermal cells when internal vascular hypertrophy or distortion occurs (Wysocka-Diller et al., 2000). This again suggests that both the development of the endodermis and C_4 BS are regulated by a non-cell autonomous signal that radiates from the internal vascular core, most likely the SHR protein.

The role of SHR in recruiting C_4 BS may also explain some of the diversity seen in Kranz anatomy. As noted above, phloem initial cells do not express the *Shr* gene and therefore must symplastically

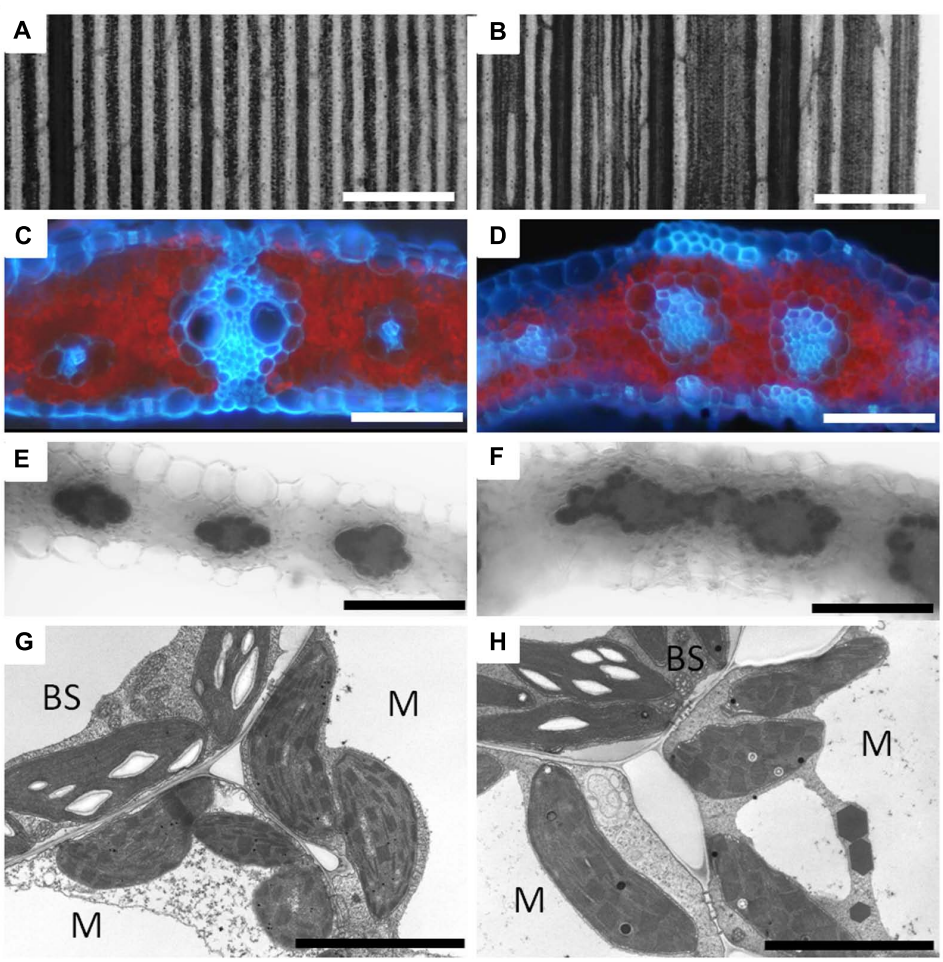

FIGURE 4 | Vascular development and bundle sheath formation in the *lsn* mutant of maize. Panel showing wild type **(A,C,E,G)** and *lsn* mutant **(B,D,F,H)** maize leaf sections. **(A)** Section of iodine potassium iodide (IKI) stained wild type leaf showing regular and uniform vascular patterning. **(B)** Section of IKI stained *lsn* mutant leaf showing distorted vascular pattering. **(C)** Cross section of wild type leaf under UV light showing canonical Kranz anatomy (red represents chlorophyll autofluorescence, blue represents autofluorescence of the cell walls). **(D)** Cross section of the *lsn* mutant under UV light, showing distorted veins with internal vascular hypertrophy and irregular internal differentiation surrounded by single layers of bundle sheath and mesophyll cells. **(E,F)** Cross sections of IKI stained leaves, respectively, showing normal starch accumulation in the BS cells and absence of staining in the M cells. **(G,H)** Transmission electron micrographs of wild type **(G)** and *lsn* mutant **(H)** BS and M cells showing normal C_4 plastid differentiation and identity in both. BS, bundle sheath cell, M, mesophyll cell, scale bars: **(A,B)** 600 μm; **(C–F)** 50 μm; **(G,H)** 5 μm.

import the SHR protein to proceed through normal phloem differentiation (Yu et al., 2010). Thus, the developing phloem presumably acts as a SHR protein sink, rather than a source of the signal. Usually, the phloem is localized within the vascular core – completely surrounded by cells that produce the SHR protein signal (Vatén et al., 2011). However, there are some C_4 species like *Atriplex rosea* which develop phloem bundles close to the edge of the vascular core in leaves (Dengler et al., 1995). In this species, the C_4 BS only encircles part of the vein, and is absent where the phloem bundle protrudes from the vascular bundle. Indeed, many other C_4 species show a similar arrangement, where C_4 BS are either absent or converted to sclerenchyma cells in the regions adjacent to the phloem bundles (Edwards and Voznesenskaya, 2011). Therefore it is reasonable to hypothesize that the internal vascular structure influences the dynamics of non-cell autonomous developmental signaling of the endodermal/BS program which could lead to the wide variations in Kranz-type structures seen in many C_4 species (Edwards and Voznesenskaya, 2011).

ENGINEERING A NOVEL FUNCTION FOR A CONSERVED TISSUE

Esau (1953) suggested that all BS in angiosperms have some endodermis-like features. But the extent to which these endodermal features are manifested in the BS varies greatly. Therefore it is likely that in C_4 plants, full Kranz anatomy arises when the underlying endodermal framework becomes enhanced – leading to the more dominant features that are associated with full endodermal/starch sheath identity. Following this reasoning, C_4 physiology may also be a manifestation of sufficient endodermal/starch sheath identity extending into the leaf. This could also explain why intermediates between C_3 and C_4 are present in some species, and why it is perceived that anatomical shifts precede physiological changes in the evolutionary trajectory toward Kranz-type C_4 (Sage et al., 2012).

How can this hypothesis for the evolution of Kranz-type C_4 be used to transfer the syndrome to C_3 plants? Again, we need to look at this issue in terms of tissue "identity" and it's functions within a plant organ. Context-dependent tissue function is common in plants. For example, it is hypothesized that in angiosperms, petals are modified leaf structures – thus they are leaves in the context of a reproductive organ (von Goethe, 1790; Pelaz et al., 2001). Therefore, the same or similar genes that usually control leaf development also impact floral development. This hypothesis has been supported experimentally. For example, the ectopic over-expression of a set of transcription factors that usually give rise to petal identity, transform leaves into petal-like structures (Pelaz et al., 2001). The change from leaf to petal tissue also transforms plastid identity from photosynthetic chloroplasts into non-photosynthetic chromoplasts. This experiment shows that entire morphology and physiology of a leaf can be reprogrammed by modulating its "identity." Most importantly, this was accomplished by altering the expression of a few transcription factors (Pelaz et al., 2001). Indeed, it seems that many aspects of tissue engineering through manipulation of developmental signaling, which is commonly used in the animal biology and medical community, may also be employed in plant anatomical and metabolic engineering.

This raises the question: can we use a similar approach, by directly manipulating tissue identity, to alter the physiology of C_3 leaves to become more C_4 like? If so, which transcription factors are the most likely targets for C_4 engineering? From the hypothesis presented in this paper, the most obvious candidates are SHR and SCR. Surprisingly, in *Arabidopsis* both SHR and SCR proteins are already present in the cells that immediately surround the vasculature in the C_3 *Arabidopsis* leaves (Wysocka-Diller et al., 2000; Gardiner et al., 2011), as they are in the developing root endodermis and in the stem starch sheath. This suggests that other interacting factors modulate the SCR/SHR complex in the C_3 BS cells and that the endodermal program is most likely regulated on the protein level. These proposed SCR/SHR interacting proteins could either confer a specific cell identity (Welch et al., 2007; Ogasawara et al., 2011), or suppress the pathway as in the case of C_3 leaves. The SCR/SHR pathway has been extensively studied in *Arabidopsis*, and yet very little has been reported on the function of these proteins in leaves (Wysocka-Diller et al., 2000; Yu et al., 2010; Gardiner et al., 2011; Ogasawara et al., 2011; Cui et al., 2012). From these data, it is reasonable to hypothesize that there may be a negative feedback loop to repress the endodermal developmental pathway in leaves. This may be why when either SHR or SCR are knocked out or over-expressed in *Arabidopsis*, major structural aspects of leaves are for the most part, unaltered (Cui et al., 2007). These reports also support the hypothesis that C_3 plants may have functional repressors in the leaves that mediate the down-regulation of the key genes needed for Kranz and C_4 differentiation irrespective of the amount of SCR or SHR protein present during development. Other cell types may share many of the developmental signaling cascades with the endodermis in the stems and petioles, but their tissue specificity may be controlled by other SHR/SCR interacting proteins that function in their respective feed-forward differentiation pathways. This may also explain why SHR and SCR are found in developing stomata and in the case of SCR, in the L1 layer of the shoot meristem (Wysocka-Diller et al., 2000; Kamiya et al., 2003; Lim et al., 2005), tissues not associated with the endodermis. This suggests that although SHR and SCR are essential for the patterning and formation of the endodermis and other cell types, they do not individually confer cell specificity or "identity." Analogous to the ABCE model of floral development (Bowman et al., 2012), specific variants of the endodermis may be under the combinatorial control of multiple factors that form a functional protein complex that regulates differentiation. In other words, SHR and SCR are essential base, or "E" type (Bowman et al., 2012) functions in the endodermal developmental program.

If SHR and SCR are not direct targets for engineering Kranz-type C4, then what genes are? From a variety of published reports, the most likely candidates to function with SHR and SCR are the interacting proteins which include, but may not be restricted to, the indeterminate-domain family of transcription factors (IDDs; Levesque et al., 2006; Welch et al., 2007; Tanimoto et al., 2008; Ogasawara et al., 2011). Within the roots and stems, different combinations of these factors promote the formation of root and stem endodermal identity, quiescent cells, and stem cells. For example, in *Arabidopsis* roots a combination of AtIDD10 and AtIDD3 maintains stem cell identity (Welch et al., 2007).

In stems, AtIDD15/SHOOTGRAVITROPISM5 (SGR5) functions with AtSHR and AtSCR to promote starch sheath identity (Tanimoto et al., 2008). But the most interesting and tantalizing evidence for the involvement of the IDDs in BS development comes from IDD over-expression studies. For example, when AtIDD8/*Nutcracker*, a target and interacting protein of AtSHR and AtSCR in the root endodermis (Levesque et al., 2006), was over-expressed in *Arabidopsis* (Seo et al., 2011), photosynthesis and plastid structures and were both dramatically altered. Most notable of these alterations is that M chloroplasts displayed reduced granal stacking – similar to what is seen in the BS of C_4 plants (Levesque et al., 2006). This finding is reminiscent to the conversion of leaves into petals where chromoplasts developed instead of chloroplasts in the petaloid-like structures (Pelaz et al., 2001) showing that physiology can be controlled by developmental programming.

Only one of the IDD genes has been characterized in a C_4 plant thus far. In maize, loss of function of *Indeterminate growth1* (*ZmID1*), the founding member of the gene family, results in altered growth and flowering time (Colasanti et al., 1998). Interestingly, *id1* mutants also have altered expression of many of the genes involved in C_4 biochemistry, suggesting there may be a broader role for ID1 in leaf development and physiology (Coneva et al., 2007). *ZmID1* is only expressed at the base of developing leaves and decreases as the leaf matures, suggesting a role in leaf development (Wong and Colasanti, 2007). In this region it is expressed in all cells. Therefore, based on overlapping expression with both *ZmScr* and *ZmShr* genes, and the altered expression of C_4-related genes in the mutant, it is likely that *ZmID1* plays a role in the development of the C_4 pathway in maize. Many of the other IDD, SHR-like and SCR-like genes in maize are also expressed at the base of the developing leaf and have either BS- or M-specific expression patterns (Li et al., 2010), suggesting potential roles in establishing C_4 BS or M cell identity and cell-specific organization of physiology. However, more research is needed to elucidate these proposed roles for *ZmID*1 and other IDD genes in either the SCR/SHR or C_4 developmental pathways in leaves.

The IDD class of genes may also have the potential to act as negative regulators of endodermal development and identity. Recently it was found that some of the members of the IDD gene family contain ethylene-responsive element binding factor-associated amphiphilic repression (EAR) domains (Wu et al., 2013), which have been shown to act as strong transcriptional repressors. Might these be the factors that keep the endodermis/Kranz program suppressed in C_3 leaves as hypothesized earlier? Overall, the published data on the IDD class of genes suggests they may play a significant role in Kranz-type C_4 regulation and development, both as potential positive and negative regulators. However, much more research is needed to explore the hypotheses presented here.

EVOLUTION OF KRANZ-TYPE C4 MECHANISM IN MONOCOTS: REVISITING THE PHYLLODE HYPOTHESIS

The emergence of C_4 in monocots appears to be ancient, arising with the grasses and sedges as they began to diverge from the other monocots (Sage et al., 2011). Can the hypothesis stated above, that Kranz-type C_4 is a synergistic interaction between the photosynthetic cells and the endodermis, also shed light on the evolution of C_4 in grass leaves? In order to explore this question, it is essential to compare eudicot and monocot leaf blade anatomy. Most important is to recognize the theory that monocot's leaves may not be true "leaf blade" tissue when compared to the eudicots (Arber, 1918; Kaplan, 1973).

It has been hypothesized that monocots evolved in an aquatic environment (Arber, 1918). This dramatically shifted the morphology of the shoot organs, such as leaves and stems. It is presumed that when these plants became submerged, their petioles or lower leaf blades became greatly extended in order to keep the leaf blades above or on the surface of the water. Over time, the upper leaf blade became greatly reduced, resulting in the petiole/lower leaf zone becoming the primary photosynthetic organ of the plant (Arber, 1918). The petiole/lower leaf zone then expanded and extrapolated into a new leaf blade (Tsiantis et al., 1999; Nardmann et al., 2004). The phyllode theory is illustrated in **Figure 5**. Whether the monocot leaf blade is derived from either the petiole as argued by Arber (1918) or the lower leaf zone (base including stipules) as argued by Kaplan (1973) is still unclear and highly debated. However, in either case, the extrapolation of either the lower leaf zone or the petiole into a new leaf base would support the arguments presented below.

The reduction of loss of a true leaf blade still occurs in some dicots. For example, the amphibious plant *Ranunculus fluitans* has different phenotypes when plants develop in dry or submerged conditions (Burkhardt, 1977). When grown on dryer soil, the plants develop similar to normal eudicots. They have broad and fully expanded leaves and are compact. However, under submerged conditions, plants reduce or eliminate the upper leaf blade tissue, and extend and expand the petiole/lower leaf zone and stems into string-like structures (Osborne, 1984), similar to leaf structures in early monocots (Arber, 1918). Indeed, these plants show extensive plasticity in their ability to dramatically shift their shoot-specific morphology and physiology. In this submerged state, the stems and petioles take over the primary role of photosynthetic organ (Kutschera and Niklas, 2009). This raises the question: what if successive generations of such amphibious plants experience the flooded situation throughout the majority of their lifecycle? Could the plants permanently fix the flooded phenotype – leading to a grass like appearance due to the reduction of the upper leaf blade and an extrapolation of the petiole/lower leaf zone and the stem (**Figure 5**)? This morphological shift reduces many of the dicot leaf blade anatomical features, the most profound being the elimination of reticulate vein patterning. The formation of parallel veins in monocots is presumed to be derived from the merger of two sides of a previously radial-organized veins in the stems and petioles (**Figure 6**; Arber, 1918; Kaplan, 1973). Alternating phloem and xylem polarity within adjacent parallel veins of some of the monocot leaves supports this view of leaf blade evolution (Arber, 1918).

This may also explain why all of the C_4 grasses and sedges use the Kranz-type mechanism (Langdale, 2011). As argued above, the petiole and lower leaf zone contains most, if not all, of the necessary anatomical and biochemical elements to establish the C_4 photosynthetic syndrome (Hibberd and Quick, 2002; Brown et al., 2010; Slewinski et al., 2012). Thus, a new leaf structure extrapolated from this area of the leaf would inherently contain all of the necessary underlying components of Kranz-type C_4.

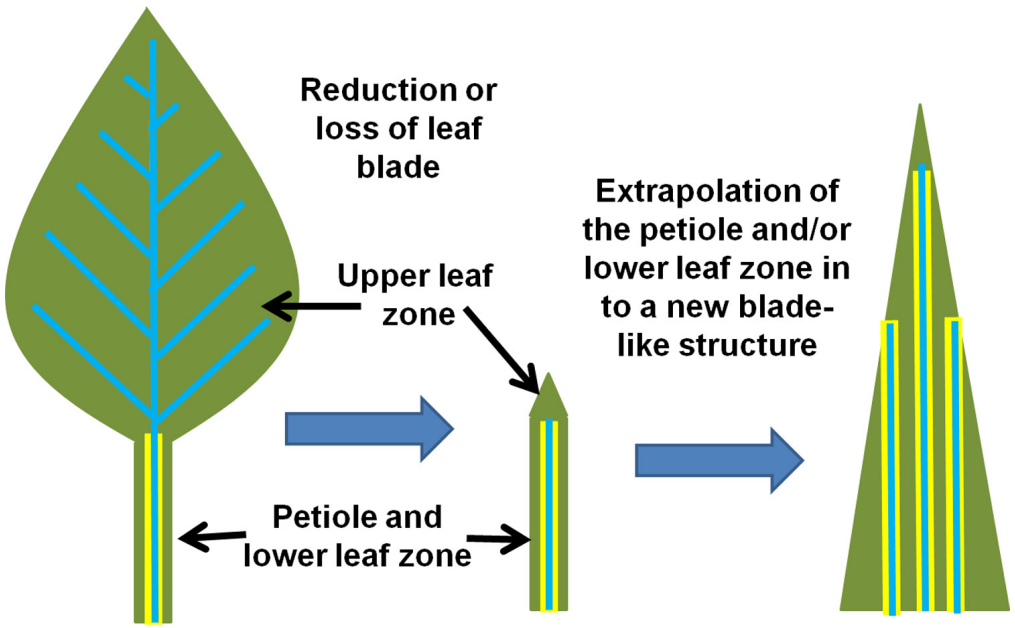

FIGURE 5 | Model for the evolution of the monocot leaf. Two major morphological shifts may have occurred that dramatically altered the monocot lineage of plants. First, the reduction or loss of a canonical dicot leaf blade (depicted on the left) resulted in the petiole/lower leaf zone structure (center) that then assumed the role of the primary photosynthetic structure. Second, the petiole/lower leaf zone expanded and extrapolated into a new "leaf blade" (right) while also extending the endodermis/starch sheath into the new photosynthetic structure. This event may have also conditioned the parallel venation that is now associated with monocot leaves (model simplified and extrapolated from Arber, 1918; Kaplan, 1973; Nardmann et al., 2004).

However, most of the monocots utilize the C_3 photosynthetic mechanism. Another look at monocot anatomy may explain why. In both C_3 petioles/lower leaf blades and in early monocots, the endodermis and the outer layer of photosynthetic cells (beneath the epidermis) are usually separated by one or many layers of non-photosynthetic parenchyma cells (**Figure 6A**; Arber, 1918). These parenchyma cells block direct interaction between the starch sheath and the active site of photosynthesis. But as monocots evolved and the grass and sedge clade emerged, leaf structures become flattened and thinner (**Figures 6B,C**). The surrounding photosynthetic layers, one on either side of the leaf, start to invade the region of the central vascular strands (Arber, 1918), most likely through the progressive elimination of parenchyma cells. In many of the grasses and sedges, these parenchyma cells are entirely absent in the leaf blade and are usually only found in the large central midvein (**Figure 6D**). This anatomical adjustment would also bring the outer photosynthetic layer of cells in direct contact with the endodermis/starch sheath, allowing the two programs to interact. Thus, the morphological shifts that lead to the emergence of the grasses and sedges could also have been the events that pre-conditioned Kranz-type C_4 within these clades.

This raises another important question. Why is rice C_3 instead of C_4? Under the phyllode theory of monocot evolution, the ancestors of rice may have been pre-conditioned for C_4 metabolism in the same manner as other C_4 grasses and sedges. However, it is important to remember that when compared to C_3 photosynthesis, the C_4 mechanism is energetically more expensive. It takes 18 ATP to fix one CO_2 molecule in the C_3 mechanism and 30 ATP in the C_4 system (Langdale, 2011). It is possible that the C_4 preconditioning event in the grasses did not confer an advantage within the environment in which the ancestors to domestic rice evolved. Thus the ancestors of rice and other C_3 grasses may have either repressed or allowed the degradation of the C_4 metabolic pathway in the leaf tissue. Under these assumptions, it can be argued that the vascular BS in rice may be a remnant of the endodermal tissue and the mestome sheath may be a remnant of the pericycle (**Figure 7**; Martins and Scatena, 2011). It is interesting to point out that it only took one mutation in the SCR gene of maize to produce many of the anatomical features that are seen in rice. Most notably, the starch-less BS cells reported in the *scarecrow* mutant of maize (Slewinski et al., 2012) have a striking resemblance to the vascular BS in rice (Langdale, 2011). Both cell types form a non-photosynthetic BS with undifferentiated plastids. Additionally, there are many other monocots that followed the same evolutionary trajectory as the grasses, producing flattened leaf blades that lack non-photosynthetic parenchyma cells, but retaining the C_3 photosynthetic mechanism.

This may be why in oat-maize addition lines, the addition of individual maize chromosomes do not confer a functional C_4 photosynthetic mechanism (Tolley et al., 2012), because the oat C_4 program may have been suppressed or undergone degradation in leaves. However, in these studies, it is also important to take into consideration the caveats of the experiment itself. For example, in wheat, maize chromosomes are destroyed after fertilization (Lefebvre and Devaux, 1996), a phenomenon that is exploited in the production of double haploid wheat and oat. Thus, the addition of individual chromosomes may not represent a true "addition"

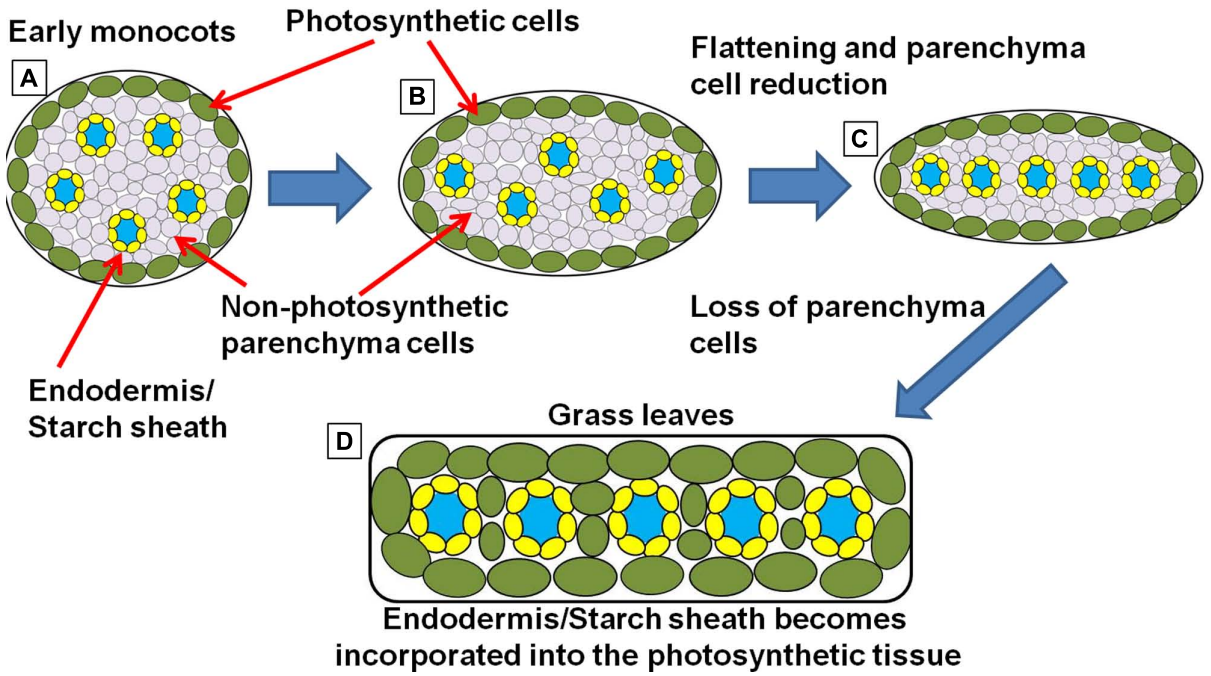

FIGURE 6 | Simplified schematic representation of cross sections through monocot "leaf blades" along the evolutionary trajectory toward the grasses. (A) Simplified model of leaf structure in the early monocots (note: the early monocot leaves are depicted as radial structures in order to simplify the concepts presented). The vasculature encased in endodermal starch sheath tissue is separated from the outer photosynthetic layer by non-photosynthetic parenchyma cells. **(B)** The leaf structure begins to flatten and compress the vascular cores toward the center of the leaf, leading to a parallel vein patterning seen in **(C)**. In grasses and sedges **(D)** the non-photosynthetic parenchyma cells are reduced or completely absent, bringing the outer photosynthetic layers in contact with the endodermis/starch sheath layer that surrounds the vasculature (model simplified and extrapolated form Arber, 1918).

of C_4 genes that can be expected to function normally. The additional alien chromosome may undergo inactivation when taken out of the context of it native genomic and cellular context. This is commonly the case when exotic chromosomes are added into animal cell lines. It is likely, as mentioned above, the underlying factors that give rise to the endodermis and starch sheath, the SHR and SCR proteins, are already present within the nuclei of cells that comprise the C_3 BS (Wysocka-Diller et al., 2000; Gardiner et al., 2011). Analogous to the C_3 BS, it is possible that the negatively regulating interacting factors are in place within rice leaves – suppressing the development of full or sufficient endodermal identity. Suppression of the C_4 pathway in hybrids between closely related C_3 and C_4 species has also been well documented (Brown and Bouton, 1993), supporting the hypothesis that C_3 plants repress the activation of sufficient endodermal program in the leaves. Thus, if the full genome complement fails to activate a C_4-like state in C_3–C_4 hybrids, it is unlikely that an individual chromosome, a partial genomic component, can initiate the C_4 program within the C_3 context.

The mechanism of C_4 suppression or down-regulation could also be argued for many of the C_3 grasses such as bamboo, oat, and wheat. Intriguingly, five independent reversals from C_4 to C_3 have been reported in the grasses (Vicentini et al., 2008). Is it possible that the ancestor at the base of the Pooideae (containing oat, barley, and wheat), Ehrhartoidea (containing rice), and Bambusoideae (containing bamboo) families underwent a C_4 to C_3 reversal early in its evolution? These three families contain only C_3 species, unlike the majority of the other grass families such as Paniceae, Andropogoneae, and Centothecoideae in which some or all of its members contain C_4 species (Vicentini et al., 2008; Sage et al., 2011). It is tempting to speculate that it is harder to re-evolve the C_4 mechanism from a C_4 to C_3 reversal species than it is to newly evolve from a basic C_3 species.

However, there does seem to be some hope for rice. When photosynthesis was surveyed in diverse rice species, considerable variation in photosynthetic rates was found (Yeo et al., 1994). None of the rice species were shown to employ the C_4 mechanism, but some varieties had unusually low photorespiration rates, as well as increased phosphoenolpyruvate carboxylase activity and photosynthetic rates that are comparable to reported C_3–C_4 intermediate species. Thus, similar to the arguments for the origin of the vascular BS, some physiological aspects of the ancient C_4 preconditioning event in the grasses may persist in a few rice species.

FUTURE PERSPECTIVES

How can we transfer the Kranz-type C_4 syndrome into C_3 crops such as soybean and rice? From the hypothesis describe in this paper, the conversion of dicot species such as soybean may be easier than previously envisioned. Isolation of both the positive and negative regulators that control endodermal development would be the first step in engineering C_4 by recapitulating

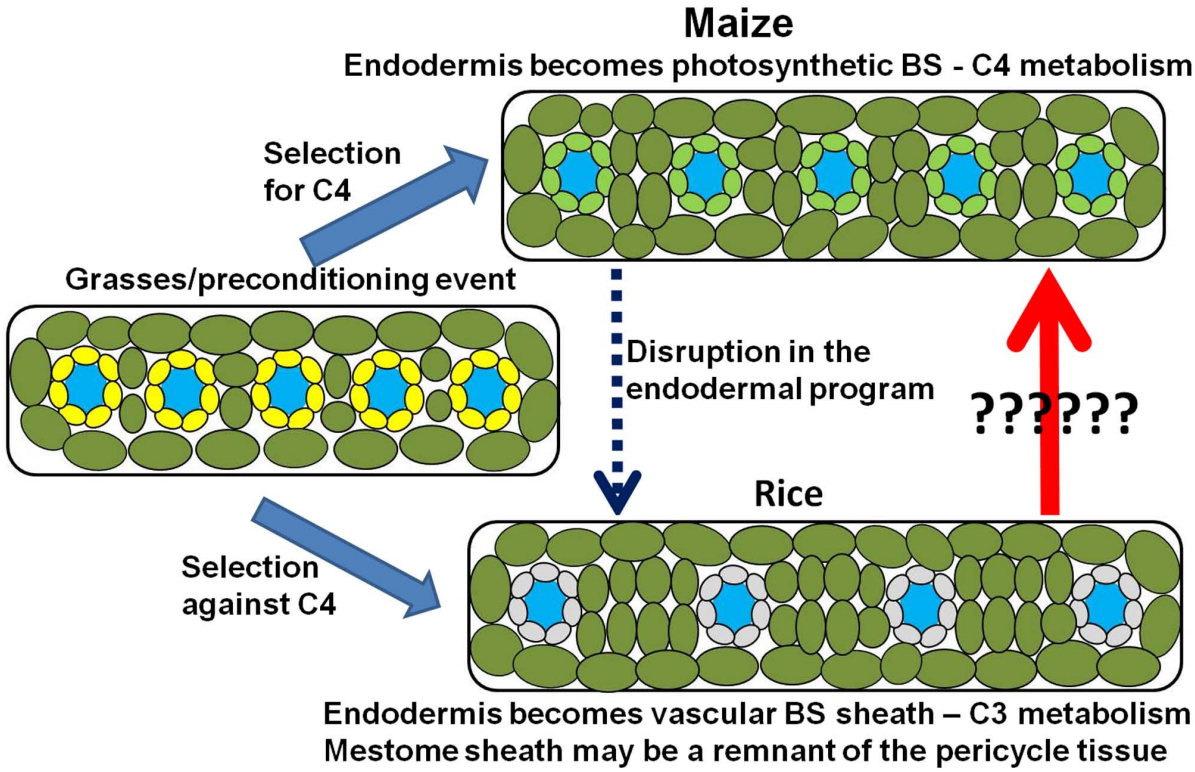

FIGURE 7 | Model for the photosynthetic divergence of rice and maize. In maize (top), the endodermis/starch sheath becomes incorporated into the photosynthetic system – giving rise to the bundle sheath (Kranz anatomy) and synergistic interaction that underlies C_4 photosynthesis. In rice (bottom), the synergistic interaction is selected against, thus maintaining a C_3 photosynthetic mechanism. However, structural remnants of the endodermis/starch sheath remain after the endodermal program is either disrupted or suppressed. The endodermis/starch sheath becomes the non-photosynthetic vascular bundle sheath in rice, whereas remnants of the pericycle tissue may be adapted to form the mestome sheath layer within the vascular core.

evolution. In the case of C_4 rice, the hypotheses and arguments made in this manuscript suggests that there may be alternative paths that might achieve this goal. Here I suggest that there might be two potential engineering trajectories. The first is to completely overhaul the physiology of the rice leaf with transgenic constructs that target the metabolism directly. The second is to try to reawaken the hypothesized C_4-like state of rice's distant past.

MATERIALS AND METHODS

PLANT MATERIALS, GROWTH CONDITIONS, AND TISSUE PREPARATION

Stocks containing the *lsn* mutation were kindly provided by Giuseppe Gavazzi at the Università degli Studi di Milano, Milan, Italy. Plants heterozygous for the mutation were self-pollinated to produce segregating families of mutant and wild type plants for analysis. *lsn* mutant and wild type plants were grown until the sixth leaf emerged. Leaves four and five were used for the analysis. Plants were grown and tissue processed, fixed, and stained for light and electron microscopy as described in Slewinski et al. (2012).

Maize lines containing the pin-formed1A-Yellow Fluorescent Protein (Pin1A-YFP) transgene were grown, prepared, and visualized as described in Slewinski et al. (2012).

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REFERENCES

Alassimone, J., Roppolo, D., Geldner, N., and Vermeer, J. E. M. (2012). The endodermis – development and differentiation of the plant's inner skin. *Protoplasma* 249, 433–443. doi: 10.1007/s00709-011-0302-5

Arber, A. (1918). The phyllode theory of the monocotyledonous leaf, with special reference to anatomical evidence. *Ann. Bot.* os-32, 465–501.

Bloemen, J., McGuire, M. A., Aubrey, D. P., Teskey, R. O., and Steppe, K. (2013). Transport of root-respired CO2 via the transpiration stream affects above ground carbon assimilation and CO2 efflux in trees. *New Phytol.* 197, 555–565. doi: 10.1111/j.1469-8137.2012.04366.x

Borlaug, N. E. (2002). Feeding a world of 10 billion people: the miracle ahead. *In Vitro Cell. Dev. Biol.* 38, 221–228. doi: 10.1079/IVP2001279

Borlaug, N. E. (2007). Sixty-two years of fighting hunger: personal recollections. *Euphytica* 157, 287–297. doi: 10.1007/s10681-007-9480-9

Bowman, J. L., Smyth, D. R., and Meyerowitz, E. (2012). The ABC model of flower development: then and now. *Development* 139, 4095–4098. doi: 10.1242/dev.083972

Brown, H. R., and Bouton, J. H. (1993). Physiology and genetics of interspecific hybrids between photosynthetic types. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44, 435–456. doi: 10.1146/annurev.pp.44.060193.002251

Brown, L. (2012). *Full Planet, Empty Plates: The New Geopolitics of Food Scarcity.* New York: Norton & Co.

Brown, N. J., Palmer, B. G., Stanley, S., Hajaji, H., Janacek, S. H., Astley, H. M., et al. (2010). C4 acid decarboxylases required for C4 photosynthesis are active in the mid-vein of the C3 species *Arabidopsis thaliana*, and are important in sugar and amino acid metabolism. *Plant J.* 61, 122–133. doi: 10.1111/j.1365-313X.2009.04040.x

Burkhardt, J. H. (1977). *The Spirit of System: Lamarck and Evolutionary Biology.* Cambridge: Harvard University Press.

Colasanti, J., Yuan, Z., and Sundaresan, V. (1998). The indeterminate gene encodes a zinc finger protein and regulates a leaf-generated signal required for the transition to flowering in maize. *Cell* 93, 593–603.

Coneva, V., Zhu, T., and Colasanti, J. (2007). Expression differences between normal and Indeterminate1 maize suggest downstream targets of ID1, a floral transition regulator in maize. *J. Exp. Bot.* 58, 3679–3693. doi: 10.1093/jxb/erm217

Cui, H., Hao, Y., and Kong, D. (2012). SCARECROW has a SHORT-ROOT independent role in modulating sugar response. *Plant Physiol.* 158, 1769–1768. doi: 10.1104/pp.111.191502

Cui, H., Levesque, M. P., Vernoux, T., Jung, J. W., Paquette, A. J., Gallagher, K. L., et al. (2007). An evolutionary conserved mechanism delimiting SHR movement defines a single cell layer of endodermis in plants. *Science* 318, 801–806. doi: 10.1126/science.1139531

Dengler, N. G., Dengler, R. E., Donnelly, P. M., and Filosa, M. F. (1995). Expression of the C4 pattern of photosynthetic enzyme accumulation during leaf development in *Atriplex rosea* (Chenopodiaceae). *Am. J. Bot.* 82, 318–327. doi: 10.2307/2445577

Edwards, G., and Voznesenskaya, E. (2011). "C4 photosynthesis: Kranz forms and single-cell C4 in terrestrial plants (Chapter 4)," in *C4 Photosynthesis and Related CO2 Concentrating Mechanisms*, Vol. 32, eds A. S. Raghavendra and R. F. Sage (Amsterdam: Springer), 29-61

Esau, K. (1953). *Plant Anatomy*. New York: Wiley & Sons.

Fedoroff, N. V., Battisti, D. S., Beachy, R. N., Cooper, P. J. M., Fischhoff, D. A., Hodges, C. N., et al. (2010). Radically rethinking agriculture in the 21st century. *Science* 327, 883–884. doi: 10.1126/science.1186834

Fedoroff, N. V., and Cohen, J. E. (1999). Plants and population: is there time? *Proc. Natl. Acad. Sci. U.S.A.* 96, 5903–5907. doi: 10.1073/pnas.96.11.5903

Fischer, R. A., and Edmeades, G. O. (2010). Breeding and cereal yield progress. *Crop Sci.* 50, S85-S98.

Furbank, R. T. (2011). Evolution of the C4 photosynthetic mechanism: are there really three C4 acid decarboxylation types? *J. Exp. Bot.* 62, 3103–3108. doi: 10.1093/jxb/err080

Gardiner, J., Donner, T. J., and Scarpella, E. (2011). Simultaneous activation of SHR and ATHB8 expression defines switch to preprocambial cell state in *Arabidopsis* leaf development. *Dev. Dyn.* 240, 261–270. doi: 10.1002/dvdy.22516

Geldner, N. (2013). The endodermis. *Annu. Rev. Plant Biol.* 64, 531–558. doi: 10.1146/annurev-arplant-050312-120050

Goldschmidt, R. (1933). Some aspects of evolution. *Science* 78, 539–547. doi: 10.1126/science.78.2033.539

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., et al. (2000). The SHORT-ROOT gene controls radial patterning of the *Arabidopsis* root through radial signaling. *Cell* 101, 555–567. doi: 10.1016/S0092-8674(00)80865-X

Hibberd, J. M., and Quick, W. P. (2002). Characteristics of C4 photosynthesis in stems and petioles of C3 flowering plants. *Nature* 415, 451–454. doi: 10.1038/415451a

Hibberd, J. M., Sheehy, J. E., and Langdale, J. A. (2008). Using C4 photosynthesis to increase the yield of rice – rationale and feasibility. *Curr. Opin. Plant Biol.* 11, 228–231. doi: 10.1016/j.pbi.2007.11.002

Kamiya, N., Itoh, J.-I., Morikami, A., Nagato, Y., and Matsuoka, M. (2003). The SCARECROW gene's role in asymmetric cell divisions in rice plants. *Plant J.* 36, 45–54. doi: 10.1046/j.1365-313X.2003.01856.x

Kaplan, D. R. (1973). The problem of leaf morphology and evolution in the monocotyledons. *Q. Rev. Biol.* 48, 437–457. doi: 10.1086/407703

Koizumi, K., Hayashi, T., Wu, S., and Gallagher, K. L. (2012). The SHORT-ROOT protein acts as a mobile, dose-dependent signal in patterning the ground tissue. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13010–13015. 10.1073/pnas.1205579109

Kutschera, U., and Niklas, K. (2009). Evolutionary plant physiology: Charles Darwin's forgotten synthesis. *Naturwissenschaften* 96, 1339–1354. doi: 10.1007/s00114-009-0604-z

Landoni, M., Gavazzi, G., Raascio, N., Vecchia, F. D., Consonni, G., and Dolfini, S. (2000). A maize mutant with an altered vascular pattern. *Ann. Bot.* 85, 143–150. doi: 10.1006/anbo.1999.1008

Langdale, J. A. (2011). C4 cycles: past, present, and future research on C4 photosynthesis. *Plant Cell* 23, 3879–3892. doi: 10.1105/tpc.111.092098

Langdale, J. A., Lane, B., Freeling, M., and Nelson, T. (1989). Cell lineage analysis of maize bundle sheath and mesophyll cells. *Dev. Biol.* 133, 128–139. doi: 10.1016/0012-160(689)90304-7

Lefebvre, D., and Devaux, P. (1996). Doubled haploids of wheat from wheat × maize crosses: genotypic influence, fertility and inheritance of the 1BL-1RS chromosome. *Theor. Appl. Genet.* 93, 1267–1273. doi: 10.1007/BF00223459

Levesque, M. P., Vernoux, T., Busch, W., Cui, H., Wang, J. Y., Blilou, I., et al. (2006). Whole-genome analysis of the short-root developmental pathway in *Arabidopsis*. *PLoS Biol.* 4:e143. doi: 10.1371/journal.pbio.0040143

Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S. L., et al. (2010). The developmental dynamics of the maize leaf transcriptome. *Nat. Genet.* 42, 1060–1067. doi: 10.1038/ng.703

Lim, J., Jung, J., Lim, C., Lee, M.-H., Kim, B., Kim, M., et al. (2005). Conservation and diversification of SCARECROW in maize. *Plant Mol. Biol.* 59, 619–630. doi: 10.1007/s11103-005-0578-y

Lunn, J. E., and Furbank, R. T. (1997). Localization of sucrose-phosphate synthase and starch in leaves of C4 plants. *Planta* 202, 106–111. doi: 10.1007/s004250050108

Martins, S., and Scatena, V. L. (2011). Bundle sheath ontogeny in Kranz and non-Kranz species of Cyperaceae (Poales). *Aust. J. Bot.* 59, 554–562. doi: 10.1071/BT11108

Morita, M. T., Saito, C., Nakano, A., and Tasaka, M. (2007). endodermal-amyloplast less 1 is a novel allele of SHORT-ROOT. *Adv. Space Res.* 39, 1127–1133. doi: 10.1016/j.asr.2006.12.020

Nardmann, J., Ji, J., Werr, W., and Scanlon, M. J. (2004). The maize duplicate genes Narrow sheath1 and Narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. *Development* 131, 2827–2839. doi: 10.1242/dev.01164

Nelson, T., and Dengler, N. G. (1997). Leaf vascular pattern formation. *Plant Cell* 9, 1121–1135. doi: 10.1105/tpc.9.7.1121

Ogasawara, H., Kaimi, R., Colasanti, J., and Kozaki, A. (2011). Activity of the transcription factor JACKDAW is essential for SHR/SCR-dependent activation of SCARECROW and MAGPIE and is modulated by reciprocal interactions with MAGPIE, SCARECROW and SHORT-ROOT. *Plant Mol. Biol.* 77, 489–499. doi: 10.1007/s11103-011-9826-5

Osborne, D. (1984). Ethylene and plants of aquatic and semi-aquatic environments: a review. *Plant Growth Regul.* 2, 167–185. doi: 10.1007/BF00124766

Pelaz, S., Tapia-López, R., Alvarez-Buylla, E. R., and Yanofsky, M. F. (2001). Conversion of leaves into petals in *Arabidopsis*. *Curr. Biol.* 11, 182–184.

Pick, T. R., Bräutigam, A., Schlüter, U., Denton, A. K., Colmsee. C., Scholz, U., et al. (2011). Systems analysis of a maize leaf developmental gradient redefines the current C4 model and provides candidates for regulation. *Plant Cell* 23, 4208–4220. 10.1105/tpc.111.090324

Reynolds, M., Bonnett, D., Chapman, S. C., Furbank, R. T., Manès, Y., Mather, D. E., et al. (2011). Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. *J. Exp. Bot.* 62, 439–452. doi: 10.1093/jxb/erq311

Reynolds, M., Foulkes, M. J., Slafer, G. A., Berry, P., Parry, M. A. J., Snape, J. W., et al. (2009). Raising yield potential in wheat. *J. Exp. Bot.* 60, 1899–1918. doi: 10.1093/jxb/erp016

Ruiz-Vera, U. M., Siebers, M., Gray, S. B., Drag, D. W., Rosenthal, D. M., Kimball, B. A., et al. (2013). Global warming can negate the expected CO2 stimulation in photosynthesis and productivity for soybean grown in the Midwestern United States.

Plant Physiol. 162, 410–423. doi: 10.1104/pp.112.211938

Sage, R. F. (2004). The evolution of C4 photosynthesis. *New Phytol.* 161, 341–370. doi: 10.1111/j.1469-8137.2004.00974.x

Sage, R. F., Christin, P.-A., and Edwards, E. J. (2011). The C4 plant lineages of planet Earth. *J. Exp. Bot.* 62, 3155–3169. doi: 10.1093/jxb/err048

Sage, R. F., Sage, T. L., and Kocacinar, F. (2012). Photorespiration and the evolution of C4 photosynthesis. *Annu. Rev. Plant Biol.* 63, 19–47. doi: 10.1146/annurev-arplant-042811-105511

Sage, R. F., and Zhu, X.-G. (2011). Exploiting the engine of C4 photosynthesis. *J. Exp. Bot.* 62, 2989–3000. doi: 10.1093/jxb/err179

Scarpella, E., Barkoulas, M., and Tsiantis, M. (2010). Control of leaf vein development by auxin. *Cold Spring Harb. Perspect. Biol.* 2, a001511. doi: 10.1101/cshperspect.a001511

Seo, P. J., Ryu, J., Kang, S. K., and Park, C.-M. (2011). Modulation of sugar metabolism by an INDETERMINATE DOMAIN transcription factor contributes to photoperiodic flowering in *Arabidopsis*. *Plant J.* 65, 418–429. doi: 10.1111/j.1365-313X.2010.04432.x

Slewinski, T. L., Anderson, A. A., Zhang, C., and Turgeon, R. (2012). Scarecrow plays a pole in establishing Kranz anatomy in maize leaves. *Plant Cell Physiol.* 53, 2030–2037. doi: 10.1093/pcp/pcs147

Sud, R. M., and Dengler, N. G. (2000). Cell lineage of vein formation in variegated leaves of the C4 grass *Stenotaphrum secundatum*. *Ann. Bot.* 86, 99–112. doi: 10.1006/anbo.2000.1165

Tanimoto, M., Tremblay, R., and Colasanti, J. (2008). Altered gravitropic response, amyloplast sedimentation and circumnutation in the *Arabidopsis* shoot gravitropism 5 mutant are associated with reduced starch levels. *Plant Mol. Biol.* 67, 57–69. doi: 10.1007/s11103-008-9301-0

Tolley, B. J., Sage, T. L., Langdale, J. A., and Hibberd, J. M. (2012). Individual maize chromosomes in the C3 plant oat can increase bundle sheath cell size and vein density. *Plant Physiol.* 159, 1418–1427. doi: 10.1104/pp.112.200584

Tsiantis, M., Schneebergerm, R., Golzm, J. F., Freeling, M., and Langdale, J. A. (1999). The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. *Science* 284, 154–156. doi: 10.1126/science.284.5411.154

Vatén, A., Dettmer, J., Wu, S., Stierhof, Y.-D., Miyashima, S., Yadav, S. R., et al. (2011). Callose biosynthesis regulates symplastic trafficking during root development. *Dev. Cell* 21, 1144–1155. doi: 10.1016/j.devcel.2011.10.006

Vicentini, A., Barber, J. C., Aliscioni, S. S., Giussani, L. M., and Kellogg, E. A. (2008). The age of the grasses and clusters of origins of C4 photosynthesis. *Glob. Change Biol.* 14, 2963–2977. doi: 10.1111/j.1365-2486.2008.01688.x

von Caemmerer, S., Quick, W. P., and Furbank, R. T. (2012). The development of C4 rice: current progress and future challenges. *Science* 336, 1671–1672. doi: 10.1126/science.1220177

von Goethe, J. W. (1790). *Versuch die metamorphose der pflanzen zu erklaren*. Gotha: C.W. Ettinger.

Wang, L., Peterson, R. B., and Brutnell, T. P. (2011). Regulatory mechanisms underlying C4 photosynthesis. *New Phytol.* 190, 9–20. doi: 10.1111/j.1469-8137.2011.03649.x

Welch, D., Hassan, H., Blilou, I., Immink, R., Heidstra, R., and Scheres, B. (2007). *Arabidopsis* JACKDAW and MAGPIE zinc finger proteins delimit asymmetric cell division and stabilize tissue boundaries by restricting SHORT-ROOT action. *Genes Dev.* 21, 2196–2204. doi: 10.1101/gad.440307

Westhoff, P., and Gowik, U. (2010). Evolution of C4 photosynthesis – looking for the master switch. *Plant Physiol.* 154, 598–601. doi: 10.1104/pp.110.161729

Wong, A. Y. M., and Colasanti, J. (2007). Maize floral regulator protein INDETERMINATE1 is localized to developing leaves and is not altered by light or the sink/source transition. *J. Exp. Bot.* 58, 403–414. doi: 10.1093/jxb/erl206

Wu, S., and Gallagher, K. L. (2012). Transcription factors on the move. *Curr. Opin. Plant Biol.* 15, 645–651. doi: 10.1016/j.pbi.2012.09.010

Wu, X., Tang, D., Li, M., Wang, K., and Cheng, Z. (2013). Loose plant architecture 1, an INDETERMINATE domain protein involved in shoot gravitropism, regulates plant architecture in rice. *Plant Physiol.* 161, 317–329. doi: 10.1104/pp.112.208496

Wysocka-Diller, J. W., Helariutta, Y., Fukaki, H., Malamy, J. E., and Benfey, P. N. (2000). Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. *Development* 127, 595–603.

Yeo, M. E., Yeo, A. R., and Flowers, T. J. (1994). Photosynthesis and photorespiration in the genus *Oryza*. *J. Exp. Bot.* 45, 553–560. doi: 10.1093/jxb/45.5.553

Yu, N.-I., Lee, S., Lee, M.-H., Heo, J.-O., Chang, K., and Lim, J. (2010). Characterization of SHORT-ROOT function in the *Arabidopsis* root vascular system. *Mol. Cells* 30, 113–119. doi: 10.1007/s10059-010-0095-y

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The role of K^+ channels in uptake and redistribution of potassium in the model plant *Arabidopsis thaliana*

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Potassium (K^+) is inevitable for plant growth and development. It plays a crucial role in the regulation of enzyme activities, in adjusting the electrical membrane potential and the cellular turgor, in regulating cellular homeostasis and in the stabilization of protein synthesis. Uptake of K^+ from the soil and its transport to growing organs is essential for a healthy plant development. Uptake and allocation of K^+ are performed by K^+ channels and transporters belonging to different protein families. In this review we summarize the knowledge on the versatile physiological roles of plant K^+ channels and their behavior under stress conditions in the model plant *Arabidopsis thaliana*.

Keywords: plant potassium channel, *Shaker*, TPK, K_{ir}-like, *Arabidopsis thaliana*, voltage-dependent, voltage-independent

INTRODUCTION

Potassium (K^+) is essential for growth and development of an organism. It is involved in various important cellular processes, like stabilization of protein synthesis, activation of enzymes, neutralization of negative charges on proteins and many more. In addition to the above mentioned tasks, in plants it is a key player in osmotic processes contributing to cellular turgor, cell elongation, translocation of photosynthates, maintenance of cytosolic pH homeostasis, and the setting of the membrane potential along with the proton motive force (Maathuis, 2009; Marschner, 2012). All these functions justify it being the most abundant inorganic cation in plants, contributing to up to 10% of their dry mass (Leigh and Wyn Jones, 1984).

Potassium is a major factor in resistance to drought, salinity, and fungal diseases (Amtmann et al., 2008). This explains why it is of crucial importance in agriculture affecting crop yield. For performing the tasks explained above, plants require potassium concentrations ranging between 100–200 mM in the cytoplasm (Wyn Jones and Pollard, 1983). In contrast, concentration of potassium in soil (10–100 μM) is 3–4 orders of magnitude lower (Schroeder et al., 1994). Therefore, a plant has to invest energy for the uptake of K^+ and its distribution throughout the plant.

The transport of potassium is accomplished by a variety of transporter proteins. In the plant model organism *Arabidopsis thaliana* a total of 71 K^+ channels and transporters have already been identified (Mäser et al., 2001; Véry and Sentenac, 2003; Amtmann et al., 2004; Wang and Wu, 2010). They have been categorized into six different gene families, comprising of three channel families and three transporter families (KUP/HAK/KT, HKT, and CPA families; Gierth and Mäser, 2007; Chanroj et al., 2012; Gomez-Porras et al., 2012).

The three identified families of K^+ channels are *Shaker*, Tandem-Pore K^+ (TPK) and K_{ir}-like channels. Recent phylogenetic data, however, evidenced that K_{ir}-like channels in fact belong to the TPK family and originated by evolutionarily recent gene duplication and partial deletion events (Marcel et al., 2010; Voelker et al., 2010; Gomez-Porras et al., 2012). We therefore do not consider K_{ir}-like channels as a separate family anymore. K^+ channels are active as multimeric proteins composed of two or four α-subunits, which are characterized by the presence of either one or two pore (P) domains. In the functional multimeric protein, four P domains are associated to form part of the conduction pathway, including its selectivity filter. K^+ selective channels have the hallmark motif TXGYGD/E in their P domains (Lebaudy et al., 2007; **Table 1**, **Figure 1**).

Abbreviations: ABA, abscisic acid; ATP, adenosine triphosphate; CBL, calcineurin B-like calcium sensor; CDPK, Ca^{2+} dependent protein kinase; cGMP, cyclic guanosine monophosphate; CIPK, CBL-interacting protein kinase; ER, endoplasmic reticulum; GFP, green fluorescent protein; P, pore; PKC, protein kinase C; PP2C, 2C-type protein phosphatase; SNARE, soluble N-ethylmaleimide–sensitive factor protein attachment protein receptor; TM, transmembrane; YFP, yellow fluorescent protein.

Table 1 | Overview of location, function and regulation of plant K^+ channels.

	Channel	Other names	Voltage dependence	Location	Function	Direct and indirect effectors		Heteromerization with	References
						positive	negative		
Shaker-like	KAT1	–	K_{in}	PM; GC (leaves, petioles)	involved in stomatal opening	pH_{ext}, ATP, 14-3-3, Auxin,	cGMP, CDPK and/or other kinase, ABA	KAT2, AtKC1	Anderson et al., 1992; Schachtman et al., 1992; Hedrich et al., 1995; Hoshi, 1995; Nakamura et al., 1995; Véry et al., 1995; Ichida et al., 1997; Li et al., 1998; Hoth and Hedrich, 1999; Berkowitz et al., 2000; Kwak et al., 2001; Pilot et al., 2001; Szyroki et al., 2001; Philippar et al., 2004; Sutter et al., 2006, 2007; Sottocornola et al., 2006, 2008; Lebaudy et al., 2010; Reuff et al., 2010; Sato et al., 2010; Gonzalez et al., 2012
	KAT2	–	K_{in}	PM; GC (leaves, petioles, stem); phloem (aerial parts)	involved in stomatal opening	pH_{ext}, Auxin	–	KAT1, AKT2, AtKC1	Pilot et al., 2001; Szyroki et al., 2001; Philippar et al., 2004; Ivashikina et al., 2005; Xicluna et al., 2007; Lebaudy et al., 2008a, 2010
	AKT1	KT1	K_{in}	PM; root, GC, hydathodes	K^+ uptake from soil; stomatal opening?	CBL-CIPK	PP2C, CBL10, AtKC1 (SNARE)	AtKC1	Sentenac et al., 1992; Basset et al., 1995; Lagarde et al., 1996; Hirsch et al., 1998; Spalding et al., 1999; Dennison et al., 2001; Szyroki et al., 2001; Xu et al., 2006; Li et al., 2006; Lee et al., 2007; Rubio et al., 2008, 2010; Honsbein et al., 2009; Geiger et al., 2009; Pyo et al., 2010; Wang and Wu, 2010; Lan et al., 2011; Ren et al., 2013
	AKT5	KT5	K_{in}	pollen	–	–	–	–	Lacombe et al., 2000b; Mouline et al., 2002
	SPIK	AKT6	K_{in}	PM; pollen	pollen tube development	pH_{ext}	Ca^{2+}_{cyt} and CDPK11/24	–	Mouline et al., 2002; Zhao et al., 2013
	AKT2	AKT2/3 AKT3 KT2/3	K_{weak}	PM; phloem; GC	K^+ battery (ensures phloem loading under energy limiting conditions); stomatal movement	ABA, kinase?	$pH_{cyt/ext}$, Ca^{2+1}_{ext}	KAT2, AtKC1	Marten et al., 1999; Deeken et al., 2000, 2002; Lacombe et al., 2000b; Dreyer et al., 2001; Szyroki et al., 2001; Chèrel et al., 2002; Ivashikina et al., 2005; Michard et al., 2005a,b; Xicluna et al., 2007; Gajdanowicz et al., 2011; Held et al., 2011; Sandmann et al., 2011
	AtKC1	KAT3 AtLKT1 KC1	regulatory subunit	PM; root; GC, hydathodes, trichomes	regulation of AKT1, and other K_{in} channels?	–	–	AKT1, AKT2, KAT1, KAT2	Szyroki et al., 2001; Reintanz et al., 2002; Pilot et al., 2003; Duby et al., 2008; Geiger et al., 2009; Honsbein et al., 2009; Grefen et al., 2010; Wang and Wu, 2010; Jeanguenin et al., 2011

(Continued)

Table 1 | Continued

	Channel	Other names	Voltage dependence	Location	Function	Direct and indirect effectors		Heteromerization with	References
						positive	negative		
	SKOR	–	K_{out}	PM; pericycle and xylem parenchyma; pollen	K^+ loading to xylem	H_2O_2	external K^+, $pH_{cyt/ext}$, ABA	GORK	Gaymard et al., 1998; Lacombe et al., 2000a; Mouline et al., 2002; Pilot et al., 2003; Dreyer et al., 2004; Johansson et al., 2006; Garcia-Mata et al., 2010
	GORK	–	K_{out}	PM; root, GC	involved in stomatal closure	H_2O_2	external K^+, $pH_{cyt/ext}$, ABA	SKOR	Ache et al., 2000; Szyroki et al., 2001; Ivashikina et al., 2001; Hosy et al., 2003; Becker et al., 2003; Dreyer et al., 2004
TPK	TPK1	KCO1	Voltage independent	VM; root; leaves; flower	K^+ homeostasis; osmoregulation; stomatal movement; germination	pH_{cyt}, GRF6 (14-3-3), Ca^{2+}_{cyt}	–	?	Deeken et al., 2003; Philippar et al., 2003; Gobert et al., 2007
	TPK2	KCO2	–	VM; root; leaves; flower	–	–	–	?	Deeken et al., 2003; Philippar et al., 2003; Voelker et al., 2006
	TPK3	KCO6	–	VM; root; flower; seeds; senescent leaves	–	–	–	?	Deeken et al., 2003; Philippar et al., 2003; Voelker et al., 2006
	TPK4	KCO4	Voltage independent	PM; pollen	potassium homeostasis; membrane voltage control of the growing pollen tube	heat	pH_{cyt}, Ca^{2+}_{ext}	?	Becker et al., 2004
	TPK5	KCO5	–	VM; senescent leaves; flower	–	–	–	?	Deeken et al., 2003; Philippar et al., 2003; Voelker et al., 2006
	KCO3[2]	–	–	VM; leaves; roots; flower; stem; hydathodes	–	–	–	–	Deeken et al., 2003; Philippar et al., 2003; Voelker et al., 2006; Rocchetti et al., 2012

All pH data refer to pH acidification. Abbreviations: K_{in}, K_{weak}, K_{out}, inwardly, weakly, outwardly rectifying potassium channel; PM, plasma membrane; VM, vacuolar membrane; GC, guard cells; CDPK, Ca^{2+} dependent protein kinase; ABA, abscisic acid; CBL, calcineurin B-like calcium sensors; CIPK, CBL-interacting protein kinase; PP2C, 2C-type protein phosphatase; cyt, cytosolic; ext, extracellular.

[1] only inward currents are affected.

[2] KCO3 very likely evolved from TPK2 by gene duplication and a subsequent deletion of one of the two pore regions.

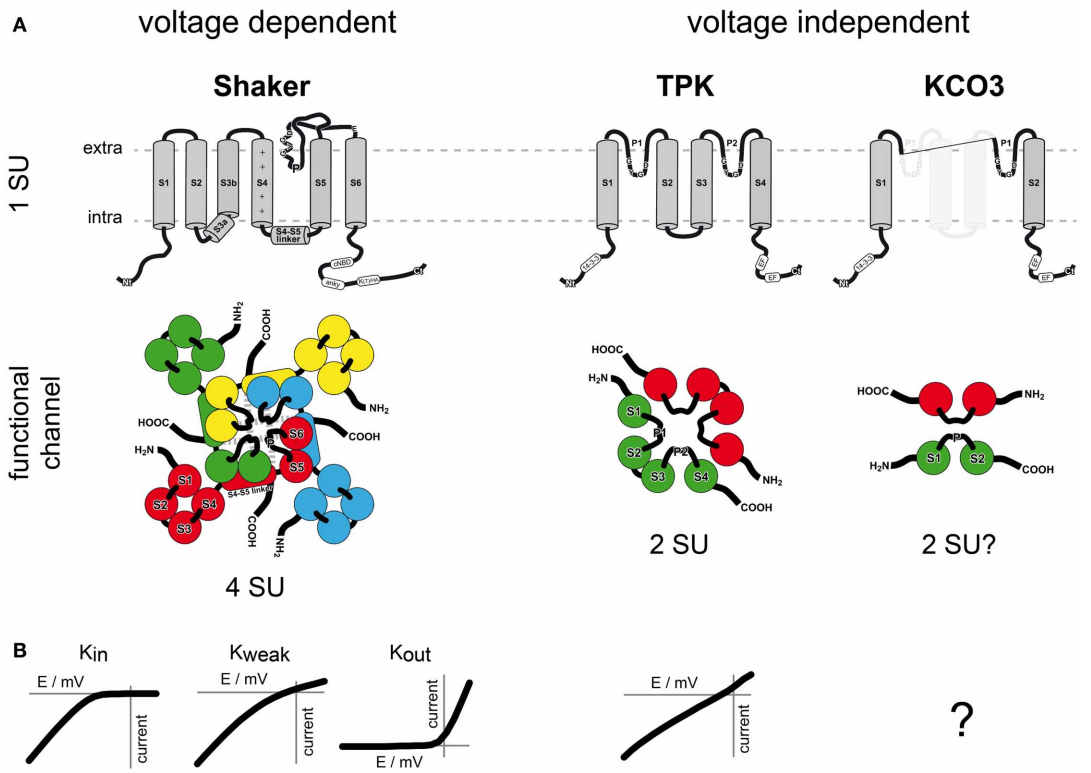

FIGURE 1 | Structure and function of K^+ channel families in plants. The two plant K^+ channel families vary in **(A)** structure and **(B)** function. *Shaker* channels form the most versatile family among plant K^+ channels. Nine members segregate into inwardly, outwardly and weakly rectifying channels. Functional channels are tetramers and operate in a voltage dependent manner. One subunit consists of six transmembrane domains (S1–S6) and one pore domain (P). The fourth transmembrane region S4 is rich in positively charged amino acids and acts together with S1, S2, and S3 as voltage sensor. Five TPK channels have been identified. One subunit contains two pore domains (P1 and P2) and two subunits are sufficient to form a functional channel. TPKs act in a largely voltage independent manner and exhibit leak like currents. KCO3 was initially classified as a K_{ir}-like channel showing two transmembrane regions and one pore domain. In fact, "plant K_{ir}-like channels" originate from TPKs by partial deletion of one selectivity-filter and two transmembrane domains. In line with this notion, only stable dimers have been detected. A K^+ transport function has not been shown for these truncated channels. Abbreviations: extra, extracellular side; intra, intracellular side; SU, subunit; +, positively charged amino acids; cNBD, cyclic nucleotide binding domain; anky, ankyrin repeat domain; $K_{(T)/HA}$, acidic domain; EF, EF hand domain.

In 1992, AKT1 and KAT1, two inward rectifying channels from *Arabidopsis* were identified by functional complementation of yeast strains defective in potassium uptake. These two members of the *Shaker*-like channel family were the first cloned plant potassium channels (Anderson et al., 1992; Schachtman et al., 1992; Sentenac et al., 1992).

In 1997, a first member of the TPK channel family was identified by *in silico* approaches, utilizing the *Arabidopsis* gene sequencing program. TPK channels are the plant counterparts of animal Tandem Pore (TWIK-like) channels (Czempinski et al., 1997). While searching for TPK1-related sequences in genome sequence database, KCO3 was identified and was thought to be structurally similar to animal potassium inward rectifying channels (Czempinski et al., 1999) leading to its initial classification into a separate family of plant K_{ir}-like channels.

VOLTAGE INDEPENDENT K^+ CHANNELS IN *ARABIDOPSIS*

TANDEM PORE POTASSIUM CHANNELS

The Tandem-Pore K^+ (TPK) channel family comprises six members (TPK1-TPK5 and KCO3, see also below for this special case) in the model plant *Arabidopsis thaliana*. TPK homologues were identified in higher plants and green algae (Voelker et al., 2010; Gomez-Porras et al., 2012). A phylogenetic analysis has shown that plant TPK channels are divided into two subfamilies: TPK1 belongs to one and TPK2, TPK3, TPK4, and TPK5 to the second subfamily. This sub division in families indicates a common ancestral origin of the channels TPK2, TPK3, TPK4, and TPK5; a hypothesis that was further supported by the analysis of chromosome segment duplication in the *Arabidopsis* genome (Marcel et al., 2010; Voelker et al., 2010; Gomez-Porras et al., 2012).

The first TPK channel (AtTPK1) was cloned via an *A. thaliana* EST database search for the conserved K^+ channel pore domain motif TXGYGD (Czempinski et al., 1997). TPKs show a TM-P-TM-TM-P-TM structure with a duplicated transmembrane-pore-transmembrane module (**Figure 1A**). In general, these channels contain one or two Ca^{2+}-binding EF hands in the cytosolic C-terminal part and binding sites for 14-3-3 proteins in the cytosolic N-terminal part, as well as a putative N-glycosylation site in the luminal loop between the pore domain and the second transmembrane domain.

Functional TPK channels are built of two of such subunits and exist as dimers (Maitrejean et al., 2011). They show a high Ca^{2+} dependency, which might be important for channel regulation (Latz et al., 2007a). TPK channels have been localized in the vacuolar membrane (Czempinski et al., 2002; Schönknecht et al., 2002). One exception is TPK4, which has been reported to localize in the plasma membrane (Becker et al., 2004; Dunkel et al., 2008). TPK4 shares 85% similarity (53% identity) with TPK5 but lacks the regulatory domains and the 14-3-3 protein interaction motif. It might thus be speculated that TPK4 evolved from TPK5 and subsequently underwent truncation events. Another exception from exclusive vacuolar localization might be TPK3. In Western blots TPK3 was also identified in thylakoid membranes (Zanetti et al., 2010) raising the question whether TPKs may have multiple subcellular locations.

Expression analysis of TPKs through quantitative real-time PCR experiments evidenced their presence in different plant tissues like roots, leaves and flowers (Deeken et al., 2003; Voelker et al., 2010). Among all TPKs, *TPK1* showed the highest expression levels in all tissues analysed, followed by *TPK3* and *TPK5*. Expression levels of *TPK2* and *TPK4* were very low. Elevated levels of *TPK2* transcripts were detected in stamen and pollen. *TPK3* transcript levels were more abundant in petals, stamen, seeds and senescent leaves.

Assembly status of Tandem-Pore K^+ channels

Promoter-reporter gene studies and qRT-PCR experiments revealed overlapping expression patterns for members of the TPK/KCO3 channel family (Czempinski et al., 2002; Deeken et al., 2003; Philippar et al., 2003; Becker et al., 2004; Voelker et al., 2006). Expression of *TPK1* overlaps with that of *TPK3* in root tips and with that of *TPK5* and *KCO3* in vascular tissues. Additionally, *TPK1*, *TPK2*, *TPK3,* and *TPK4* express in pollen. The overlapping expression patterns and their common localization in the tonoplast propose that heteromeric channel subunit combination might occur under different developmental stages or physiological conditions (Latz et al., 2007a).

Dimerization of TPK channels has been shown experimentally by using velocity sucrose gradient centrifugation of leaf homogenates expressing TPK1-GFP. This confirms the contribution of four pore domains to the K^+ selectivity filter of the TPK1 channel (Maitrejean et al., 2011). Using the same technique, AtKCO3 and AtKCO3-GFP have been observed to exist as dimers, too. These channels would thus have only two pore domains in a dimerized state (**Figure 1A**), which is not considered to be sufficient for an active, K^+-selective channel (Rocchetti et al., 2012).

With the aim of studying the assembly status of TPK/KCO family members, various experiments have been performed employing techniques like FRET and BiFC (split-YFP). Results from these approaches indicated the existence of homomeric TPK/KCO3 channels, as e.g., in the case of TPK1 or TPK5 (Voelker et al., 2006). However, so far no evidence for heteromeric channel formation could be provided. Nevertheless, there are neither convincing data ruling out this possibility. Thus it cannot be excluded that *in vivo* heteromeric channel formation might occur under different developmental and physiological conditions.

Localization of Tandem-Pore K^+ channels

In a first approach to detect the subcellular localization, the TPK1 channel has been stably over-expressed in tobacco BY-2 cells. After protein fractionation with a sucrose gradient, this K^+ channel was found to co-fractionate with tonoplast markers, giving a first clue of its localization on the vacuolar membrane (Czempinski et al., 2002). Further localization studies were performed by creating GFP fusion constructs followed by their transient expression in *A. thaliana* protoplasts. Such experiments demonstrated vacuolar localization of TPK1, TPK2, TPK3, and TPK5 (Voelker et al., 2006). In contrast, when a TPK4:GFP fusion construct was expressed in onion epidermal cells, it was found to localize partially in the plasma membrane. A major fraction, however, was detected in the ER (Becker et al., 2004; Dunkel et al., 2008). This might be either due to ER-retention or may indicate that besides TPK3, been found in the tonoplast and in the thylakoid membrane, also TPK4 may exhibit at least a dual localization profile.

Unfortunately till now, no general targeting sequence is known that "guides" TPK channels to the appropriate membrane (Vitale and Hinz, 2005; Dunkel et al., 2008). With the purpose of identifying the sorting signal of vacuolar TPK channels, various chimeras were generated between TPK4 (plasma membrane protein) and TPK1 (tonoplast protein). It is not handed down why this particular pair has been chosen and not the "twins" TPK5 and TPK4; TPK4 sharing 85% of similar amino acids with TPK5. Nevertheless, the chimeras showed that complete replacement of the cytosolic C-terminus of TPK1 results in ER retention. Further detailed analysis indicated that the terminal 25 amino acids are not important for the trafficking process. An analysis of amino acids 292–308 in the C-terminus of TPK1 could identify three diacidic motifs. Out of these three motifs, mutations in (D296G/E298G) resulted in ER-stuck TPK1 proteins, suggesting that this diacidic motif is crucial for the export of TPK1 from the ER (Dunkel et al., 2008; Voelker et al., 2010). A related study on rice TPKs identified amino acids in the cytosolic C-terminal domain that determine differential targeting of TPKs to the endomembranes of the large central lytic vacuole or of protein storage vacuoles (Isayenkov et al., 2011a) indicating a general role of certain regions in the cytosolic C-terminus for channel targeting.

Retention of TPK1 channel protein in the ER also occurred when plant leaves were treated with Brefeldin A, a fungal toxin which causes redistribution of Golgi membranes. From this observation it was inferred that the transport of TPK channel proteins to the vacuolar membrane is through a Golgi-dependent pathway and that the Golgi apparatus is the first compartment crossed by the protein after it leaves the ER (Dunkel et al., 2008). Experiments in rice indicated a more complex situation of TPK targeting. TPKs targeted to the lytic vacuole indeed cross the Golgi apparatus. However, TPKs targeted to protein storage vacuoles apparently reach the endomembrane in a Golgi-independent way (Isayenkov et al., 2011a).

Regulation and function of Tandem-Pore K^+ channels

At present the knowledge on function and regulation of plant TPKs is limited. Research is often fuelled by comparison with

related channels from other kingdoms. Animal two-pore channel activity has been shown to be regulated by interacting 14-3-3 proteins (Rajan et al., 2002). Also in plants down-regulation of K^+ channel activity in the tonoplast has been observed to be caused by interaction with 14-3-3 proteins. In TPKs, the cytosolic N-terminus comprises a classical binding motif for 14-3-3 proteins (RSXpS/pTXP) [1]. Phosphorylation of these serine or threonine residues is crucial for the interaction with 14-3-3 proteins (Latz et al., 2007a).

TPK channels are proposed to be involved in the K^+ homeostasis of plant cells by allowing the controlled intracellular K^+ transport from and into organelles. Recent experiments employing the patch clamp technique have demonstrated a mechanosensitive nature of TPK channels suggesting especially a role in osmoregulation. This concept was further supported by protoplast disruption assays (Maathuis, 2011) and seedling germination tests (Gobert et al., 2007).

AtTPK1 is ubiquitously expressed in *A. thaliana*. Using promoter-reporter gene (GUS) fusion, *TPK1* promoter activity was observed in root cortex, vascular tissue, mesophyll cells, guard cells and pollen grains (Czempinski et al., 2002). When expressed in yeast, TPK1 has characteristics of K^+-selective channels from *Vicia faba* (VK channels) previously characterized *in vivo* with strong selectivity for K^+ over Na^+ (Bihler et al., 2005; Gobert et al., 2007; Latz et al., 2007b). The activity of TPK1 is independent of the membrane voltage but was shown to be dependent on the cytosolic pH with a maximum open probability at pH 6.7, decreasing 20–30% at physiological pH 7.5–7.8. It is activated by cytosolic Ca^{2+}, remarkably exhibiting the highest affinity for calcium ions among the proteins tested including calmodulin. Interaction of TPK1 with the 14-3-3 protein GRF6 (General Release Factor 6) increases the channel activity in a dose dependent manner. This interaction does not play any role in targeting of the protein to the tonoplast (Latz et al., 2007a). All these data indicate that TPK1 is tightly controlled by cellular signals. TPK1 has been reported to participate in vacuolar K^+ release during stomatal closure and also during seed germination and radicle growth (Gobert et al., 2007).

AtTPK4 is an instantaneously activating, K^+ selective channel that is also found in the plasma membrane when expressed in *Xenopus* oocytes and yeast. *In planta*, *TPK4* exhibits low transcript abundance. It is predominantly expressed in pollen, as observed by promoter-GUS fusion analysis. TPK4 is blocked by extracellular Ca^{2+} and is insensitive toward changes in extracellular pH, but it is efficiently blocked by cytosolic acidification. Activation of TPK4 by heat has also been reported (Becker et al., 2004). TPK4 is proposed to contribute to the K^+ conductance of the pollen tube plasma membrane, where it operates as a so called "open rectifier" with saturating current at depolarizing membrane potentials.

AtTPK5 is targeted to the tonoplast. At the mRNA level, *TPK5* shows higher abundance in senescent leaves and petals (Voelker et al., 2010). Promoter GUS studies of *TPK5* have shown expression in the vascular tissues of leaves, roots, hydathodes, floral tissues and stems. *TPK5* transcript level is increased or decreased in response to external factors.

Recently AtTPK1, AtTPK2, and AtTPK5 were functionally characterized in *Escherichia coli*. The three isoforms were able to complement the K^+ uptake deficient *E. coli* mutant LB2003 on low K^+ medium (Isayenkov and Maathuis, 2013). Interestingly, in the same experiments AtTPK3 could not complement LB2003. This may indicate that this channel might be active in a different membrane environment, as for instance the thylakoid membrane (Zanetti et al., 2010).

Different isoforms of Tandem-Pore K^+ channels

Tandem-pore K^+ channels have also been identified and characterized in plant species other than *A. thaliana*, for example *Hordeum vulgare, Nicotiana tabacum, Solanum tuberosum, Oryza sativa* (Czempinski et al., 1999; Hamamoto et al., 2008a,b; Isayenkov et al., 2011a,b). It is fascinating to see that NtTPK1 from tobacco exhibits properties different from other plant TPK channels, since it is active even in the absence of Ca^{2+}. Nevertheless, increase in cytosolic Ca^{2+} resulted in an up to two fold increase in the K^+ current amplitude (Hamamoto et al., 2008a). Its current profile shows an instantaneous and a time-dependent component (Hamamoto et al., 2008b). The most interesting distinguishing feature is that two of the four identified isoforms in *N. tabacum* do not contain the conserved TXGYGD motif in the second pore domain. Instead, NtTPKb and NtTPKc possess VHG or GHG, respectively.

PLANT K_{ir}-LIKE CHANNELS

Plant K_{ir}-like channels were initially classified as an own group although they are similar to TPK channels. To date, they have been found only in the genus *Arabidopsis, (A. thaliana* and *A. lyrata*; Gomez-Porras et al., 2012). Thus, they apparently emerged just recently in evolution. Phylogenetic analyses indicated them to have originated from gene duplication of an TPK channel gene followed by a partial deletion event that resulted in the loss of one pore domain (**Figure 1A**; Marcel et al., 2010; Voelker et al., 2010). As a consequence, a plant K_{ir}-like channel subunit contains only two TM and one P region. Based on that structural feature it was speculated that plant K_{ir}-like channels are tetramers. This concept, however, is rather questionable. The genome of *A. thaliana* contains only one gene (called KCO3) coding for a K_{ir}-like subunit. Recently, KCO3 could be detected only as stable dimer at the biochemical level (Rocchetti et al., 2012) pointing further to its origin from TPK channels. Very low transcript abundance has been observed for *KCO3*. Promoter-GUS fusion constructs for *KCO3* show expression in vascular tissue of leaves, roots, flower tissue and stem and also in hydathodes as seen also for *TPK5*. KCO3 might play a role in osmoregulation, as the knock-out plant for the *KCO3* gene shows reduced growth under osmotic stress condition. However, this change in the plant phenotype can be complemented by expressing a mutant *KCO3* gene with an inactive pore region. These results indicate that the function of KCO3 under osmotic stress conditions is independent of its ability to transport potassium ions (Rocchetti et al., 2012). In conclusion, based on the current knowledge, plant K_{ir}-like

[1]pS/pT indicate the potential phosphorylation of the serine or threonine residue, respectively.

channels should be re-integrated into the TPK family, instead of being considered as a separate channel family. Their occurrence in *Arabidopsis*, only, may suggest that they just are "a freak of nature" without fundamental physiological importance outside this genus.

VOLTAGE DEPENDENT K^+ CHANNELS IN *ARABIDOPSIS*

The so-called plant *Shaker*-family is a group of voltage gated K^+ channels. In *A. thaliana* it comprises nine members. This group can be divided into three subfamilies regarding their response to the membrane voltage (Lebaudy et al., 2007; Dreyer and Blatt, 2009). Six members activate upon membrane hyperpolarization and are closed when the driving force for potassium is outwardly directed. As a consequence they elicit only inward K^+ currents (K_{in}). Two members activate upon membrane depolarization. They are closed when the driving force for potassium is inwardly directed. Thus, they elicit only outward K^+ currents (K_{out}). And one member exhibits weak voltage dependence and can mediate both, K^+ efflux and K^+ influx (K_{weak}; **Figure 1B**).

Functional plant *Shaker* channels are built of four α-subunits. Each α-subunit contains six transmembrane domains and one pore domain between the fifth and the sixth transmembrane domain. The C-terminus contains various regulatory elements, like the cyclic nucleotide binding domain, an ankyrin repeat domain, the acidic domain K_{HA} and in K_{in} channels the K_T domain (Sentenac et al., 1992; Ehrhardt et al., 1997; Gaymard et al., 1998; Dreyer et al., 2004). Besides being functional as homotetramers, the formation of heterotetramers is common and proven to occur in plants (Dreyer et al., 1997; Lebaudy et al., 2008a).

Versatile physiological roles of plant *Shaker* channels were identified in numerous experiments. Knock-out and overexpressing mutant plants, as well as heterologous expression systems like *Saccharomyces cerevisiae, Xenopus laevis* oocytes, HEK293, COS, or Sf9 cells were used to study the functionality of K^+ channels (Dreyer et al., 1999). The physiological roles and impacts of plant *Shaker* channels on the plant are described in the following sections.

K^+ UPTAKE INTO ROOTS VIA AKT1

Various conditions necessitate different uptake systems

K^+ uptake from soil is performed by a well-organized system of transport proteins each contributing in its own manner (Alemán et al., 2011). All uptake systems together operate on a broad range of K^+ concentrations and are part of an extensive regulatory network (**Figure 2**). As main K^+ uptake systems in *Arabidopsis* roots the *Shaker*-like K^+ channel AKT1 and the K^+ transporter AtHAK5 have been identified (Hirsch et al., 1998; Gierth et al., 2005; Rubio et al., 2008). At external K^+ concentrations below 0.01 mM the proton-driven H^+/K^+ co-transporter AtHAK5 is the only system responsible for K^+ uptake from the soil. At K^+ concentrations between 0.01 mM and 0.05 mM AtHAK5 and AKT1 together contribute to K^+ uptake. At higher external K^+ concentrations, AKT1 together with other unknown low affinity K^+ uptake systems are responsible for K^+ uptake from the soil (Rubio et al., 2010; Pyo et al., 2010; Caballero et al., 2012).

AKT1 and AtHAK5 are affected by different environmental conditions. Both transport proteins work at different K^+ concentration spectra and exhibit individual sensitivity toward other ions. For instance, AtHAK5 is sensitive to ammonium (NH_4^+) whereas AKT1 remains unaffected in the presence of NH_4^+. On

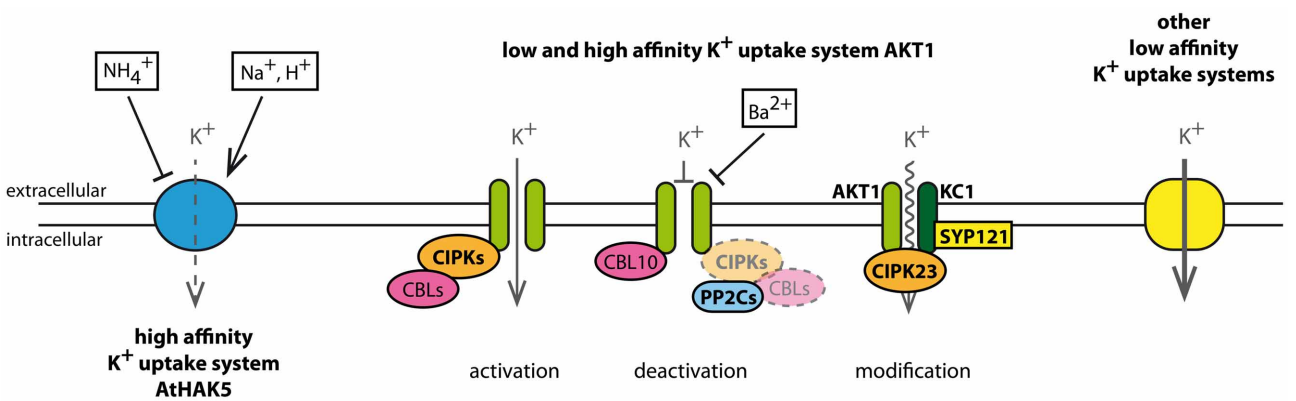

FIGURE 2 | K^+ uptake into *Arabidopsis* roots and its regulation. Depending on the actual K^+ concentration in the soil different low or high affinity K^+ uptake systems are active. At K^+ concentrations below 0.01 mM only the high affinity transporter AtHAK5 is active. It is blocked by extracellular NH_4^+ and stimulated by extracellular Na^+ and H^+. The *Shaker*-like K^+ channel AKT1 is involved in high and low affinity K^+ uptake. It is a target of an extensive regulatory network that includes calcium sensors (CBLs), kinases (CIPKs), phosphatases (PP2Cs), and the ability to form heterotetramers with AtKC1. In the presence of CBL1 or CBL9, CIPK23 phosphorylates and activates AKT1. The interaction of CIPK 6, 16, and 23 each with CBL1, 2, 3, and 9 and its effect on AKT1 were shown in yeast two-hybrid assays and *Xenopus laevis* oocytes (Lee et al., 2007). AKT1 is deactivated by a direct interaction with CBL10, external Ba^{2+}, or dephosphorylation via PP2C phosphatases. Phosphatases act directly on AKT1 or on the CIPK-CBL machinery to inactivate AKT1 (Lan et al., 2011). Furthermore, AKT1 is able to form heterotetramers with AtKC1. The heteromeric channel exhibits changed gating and permeation properties that block efficiently potential K^+ release under low external K^+ concentrations (Geiger et al., 2009). In addition, an interaction of CIPK23 with the heteromeric AKT1-AtKC1 was suggested and the contribution of SYP121 to the native characteristics of AKT1-AtKC1 was described (Honsbein et al., 2009).

the contrary, Ba^{2+} blocks AKT1 while AtHAK5 remains unaffected, and Na^+ and H^+ stimulate activity of AtHAK5 (Hirsch et al., 1998; Spalding et al., 1999; Rubio et al., 2008). Therefore, the different K^+ uptake systems complement one another and even permit K^+ uptake when one uptake system is disabled. AKT1 provides hence an alternative K^+ uptake system to the NH_4^+ sensitive AtHAK5 under low K^+ conditions.

Regulation of AKT1

AKT1 itself contributes to high and low affinity K^+ uptake and is target of a regulatory network. Xu et al. and Li et al. showed in 2006 that CIPK23 and CBL1 or CBL9 are required to activate AKT1. The two calcineurin B-like calcium sensors CBL1 and CBL9 bind to the CBL-interacting protein kinase CIPK23, which then in turn phosphorylates AKT1. All three components (AKT1-CIPK23-CBL1/9) are essential for a functional expression of AKT1 in oocytes of *X. laevis*.

Shortly after, further components of this highly complex and flexible regulatory network were discovered. Besides several CIP kinases a 2C-type protein phosphatase (PP2C), AIP1, was shown to bind and inactivate AKT1 (Lee et al., 2007). Subsequent studies detected more interrelations between several CBLs and CIPKs with AKT1 (Lee et al., 2007; Lan et al., 2011; Ren et al., 2013). Lan et al. suggested that PP2C phosphatases also interact with the CIPK-CBL complex to inhibit the phosphorylation activity of the kinase and to dephosphorylate AKT1. And Ren et al. (2013) demonstrated that CBL10 directly binds AKT1 and diminishes its activity in a concentration-dependent and CIPK-independent manner.

Many different associations of AKT1 with CBLs, CIPKs, and PP2Cs have been reported. Grefen and Blatt (2012) argue that the method used to investigate interactions between proteins, positioning of tags and the way of analysis have a decisive impact on detectable interactions. Nevertheless, CBLs, CIPKs and PP2Cs provide a comprehensive system to regulate the K^+ uptake mediated by AKT1. Especially, as different CBLs are involved in different signaling pathways this phosphorylation-dephosphorylation system provides a powerful regulatory network for the plant to respond to a broad range of environmental changes (for review see Kudla et al., 2010).

Internal regulation via heteromerization

Besides the regulation by kinases and phosphatases another member of the *Shaker*-like family alters the functionality of AKT1: AtKC1. AtKC1 is known as regulatory or silent α-subunit of K_{in} *Shaker*-like channels as it shows no currents in *Xenopus* oocytes when expressed alone and affects only K_{in} channels (Dreyer et al., 1997; Jeanguenin et al., 2011). Nevertheless, its participation in K^+ uptake and its connection to AKT1 has been recognized since long (Reintanz et al., 2002; Pilot et al., 2003). Duby et al. (2008) demonstrated AtKC1′s impact on AKT1. They described that AtKC1 shifts the activation threshold of AKT1 toward more negative values. This in turn would avert K^+ efflux through AKT1 under unfavorable conditions. The reduction of potential outward currents prevents the plant from K^+ loss under low K^+ concentrations. However, the cost of such "a valve" is a reduced channel activity that in turn implies decreased K^+ influx under more favourable conditions. Geiger et al. (2009) supported, further broadened and fine-tuned this "valve" hypothesis. They showed in electrophysiological experiments the effect of AtKC1 on AKT1 inward and outward currents under varying K^+ concentrations. Besides affecting the activation threshold, also the K^+ dependent stability of the pore has been altered in AKT1-AtKC1 heteromers. When the external K^+ concentration drops, the permeation pathway of K^+ channels gets instable and collapses (Zhou et al., 2001). The threshold concentration, below which this happens, appears to be a characteristic feature of each channel. Geiger et al. (2009) found that the pore of AKT1-AtKC1 heteromers collapses at higher K^+ concentrations than that of AKT1 homomers. Thus, heteromers comprise a more efficient block of the K^+ passage in the unfavorable outward direction.

On top of that, the association of CIPK23 with the heteromeric AKT1-AtKC1 channel has been suggested from interaction analyses in yeast (Grefen and Blatt, 2012) along with an impact of the membrane vesicle trafficking SNARE protein SYP121 (Honsbein et al., 2009). In contrast to CIPK23, SYP121 binds only to the AtKC1 α-subunit but not to the AKT1 α-subunit. SNARE proteins are involved in vesicle targeting and fusion. Thus, K^+ transport is not only regulated via the channel activity but also by membrane trafficking processes. Interestingly, the transcript level of AKT1 is constant under different environmental conditions (Lagarde et al., 1996; Pilot et al., 2003). But, the expression levels of its regulators change according to environmental stimuli (Pilot et al., 2003, review: Batistic and Kudla, 2004; Tripathi et al., 2009).

OUTWARD RECTIFIERS IN ROOTS

GORK in root hairs

Alongside the inward rectifying K^+ channel AKT1, the outward rectifying K^+ channel GORK is expressed in root epidermal cells (Ivashikina et al., 2001). GORK activates upon membrane depolarization and its gating depends on the extracellular K^+ concentration. Environmental changes in the surrounding of root hairs can appear rapidly and in response the membrane depolarizes (Càrdenas et al., 2000). GORK activates under these conditions and is considered to initiate the repolarization of the membrane. By controlling the membrane potential and the turgor in root hairs, the plant can react on environmental changes, like absence or abundance of water that cause changes in solute concentrations and affect the mechanical stability and the hydration status of the root. Furthermore, the ability of GORK to sense the extracellular K^+ concentration is supposed to enable the root hair to sense and flexibly react on the K^+ content in the soil.

Root to shoot communication via SKOR

K^+ is transported from roots to the upper parts of the plant via the xylem. The outward rectifying *Shaker*-like channel SKOR is expressed in the pericycle and the xylem parenchyma in roots. SKOR was identified as transport protein responsible for loading K^+ to the xylem based on the finding that its disruption strongly reduced the K^+ content in the shoot while the K^+ content in roots remained unaffected (Gaymard et al., 1998).

In addition to the membrane voltage, SKOR is modulated by the external K^+ concentration. In the presence of ample external K^+, the channel needs a higher membrane voltage to open and

thus minimizes the risk to serve as an undesirable K^+-influx pathway. Such behavior is achieved by a complex interplay between the pore region and the last transmembrane domain of the channel that is responsible for final channel opening and closure. When the external K^+ concentration is high, the pore region is quite rigid and strongly interacts with the last transmembrane domain of the channel. As a consequence the channel is stabilized in a closed state. Under low external K^+ conditions the pore region is less occupied by K^+ ions. As a consequence, the pore is more flexible and does not interact with the surrounding transmembrane domains anymore. Opening of the channel is possible with less energy input, i.e., at less positive membrane voltages. If the last transmembrane domains rearrange and unclench the conduction pathway, intracellular K^+ ions can re-enter the pore, stabilize it in a permeable conformation and thus enable a K^+ outward current (the K^+-sensing mechanisms has been animated in the supplementary material of Johansson et al., 2006).

K^+ distribution is also influenced by factors that are involved in stress signaling. SKOR expression is inhibited by abscisic acid (ABA). It was proposed that the reduced K^+ release to the xylem in response to ABA could be a possibility to adjust osmotic conditions by roots in stress situations (Gaymard et al., 1998). Besides, intra- and extracellular acidification negatively affects the SKOR currents. As the regulation via ABA appears on the transcriptional level, the pH sensitivity might be a complementary process to prevent K^+ loss from roots toward the shoot tissue (Lacombe et al., 2000a).

Hydrogen peroxide (H_2O_2) exhibits a contrary effect on SKOR currents. Reactive oxygen species function as signal and regulator in plant development and in responses to environmental stress situations (Torres and Dangl, 2005; Gapper and Dolan, 2006). Treatment with H_2O_2 leads to an increase in SKOR outward currents and a decrease in its half activation time (Garcia-Mata et al., 2010). This finding points to a relation between reactive oxygen species and K^+ partitioning during developmental processes and stress responses.

PHLOEM-ALLOCATION AND RETRIEVAL

Once loaded into the xylem, K^+ circulates within the whole plant. There, other K^+ channels contribute to the further distribution. The *Shaker*-like potassium channel AKT2[2] is mainly expressed in the vascular tissue of aerial parts and in guard cells of plants. However, it is not expressed until the plant is widely independent of carbohydrates provided by the seed (Marten et al., 1999; Deeken et al., 2000; Lacombe et al., 2000b; Szyroki et al., 2001; Ivashikina et al., 2005).

Charging and using the potassium battery

As the only member of the *Shaker*-like channels in plants, AKT2 features a unique channel property and can mediate both, inward and outward K^+ currents. AKT2 is in fact a specialized inward rectifying channel that can be changed into a non-rectifying channel. It exhibits two phosphorylation status-dependent gating modes that are inter-convertible (Dreyer et al., 2001; Chèrel et al., 2002; Michard et al., 2005a,b). The non-phosphorylated AKT2 (mode 1) is lacking its outward component and behaves like an inward rectifying channel. In contrast, the phosphorylated AKT2 (mode 2) is permanently open and able to conduct K^+ in the inward and in the outward direction. Two serine residues located near the intracellular side of the channel are identified as targets for phosphorylation (Michard et al., 2005a). Nevertheless, it is proposed that the two phospho-serine residues alone are not sufficient to completely convert AKT2 between its modes. Sandmann et al. (2011) proposed rather a transition via a cascade of post-translational (so far unknown) modifications. This hypothesis is fuelled by experimental observations. A lysine within the voltage sensor enables AKT2 to sense its phosphorylation status and to change between the two modes. Replacement of the lysine by serine or arginine keeps AKT2 in its inward rectifying mode 1 (Michard et al., 2005b; Sandmann et al., 2011).

Summing up, AKT2 can modulate the membrane voltage by switching between its modes of an inward or a non-rectifying channel, respectively, and phosphorylation acts as a tool for fine tuning (Deeken et al., 2002; Michard et al., 2005a,b). Gajdanowicz et al. (2011) embedded AKT2 as a central player in a "potassium battery" model in which K^+ serves as mobile energy source in vascular tissues. In source tissues, the plant invests energy to load K^+ into the phloem sieve element companion cell complexes. The loaded potassium is then circulating with the phloem stream. Under energy limiting conditions, the AKT2 channel can be switched from its inward-rectifying to its non-rectifying mode and thus enables a passage for K^+ efflux. This in turn enables the use of the K^+ gradient between the phloem and the apoplast for the reloading of photoassimilates into the phloem. This "potassium battery" concept is illustrated in the supplementary material of Gajdanowicz et al. (2011). Limiting conditions occur for example under ATP shortage or when the H^+-ATPase is down-regulated by cellular signals. The normally used H^+ gradient is then complemented by the K^+ gradient. Besides tapping the "battery," the AKT2 channel is also proposed to charge it depending on its actual gating mode (Michard et al., 2005b).

Further effects on AKT2

In addition to the gating mode modulations, AKT2 was also demonstrated to act on diverse signals involved in stress responses. The expression level of AKT2 increases in the presence of ABA, light and CO_2 assimilates (Deeken et al., 2000; Lacombe et al., 2000b). Primarily, the influences of the last two factors led to the view that AKT2 plays a role in phloem transport.

Macroscopic K^+currents mediated by AKT2 are modulated by changes in internal and external pH and external Ca^{2+} (Marten et al., 1999; Lacombe et al., 2000b). While external Ca^{2+} blocks inward currents at negative voltages in a voltage-dependent manner, acidification on both sides of the membrane diminishes AKT2 currents in the whole voltage range. Changes in pH and Ca^{2+} do not affect the gating mode of the channel indicating that H^+ and Ca^{2+} affect only the permeation pathway of AKT2. The sensitivity of AKT2 toward Ca^{2+} was investigated in guard cells.

[2]In literature, the gene encoded by the locus At4g22200 has been named AKT2, AKT3 and AKT2/3. To avoid confusions, we will summarize the data under the name AKT2 irrespective of the alternative names used in the original publications.

Ivashikina et al. (2005) showed in experiments on guard cell protoplasts that the Ca^{2+} sensitivity of K^+ uptake channels correlates with the presence of AKT2 subunits.

Recently, Held et al. (2011) demonstrated the association of AKT2 with CIPK6 and CBL4 and the effect of this assembly on macroscopic AKT2 currents. In contrast to the AKT1-CIPK-CBL complexes, no phosphorylation events could be detected *in vitro* so far. Held et al. therefore proposed for these findings a Ca^{2+} dependent targeting of AKT2 to the plasma membrane that depends solely on the physical interaction of AKT2 with CIPK6/CBL4 rather than a regulation of the channel via phosphorylation.

GUARD CELLS AND ITS K^+ CHANNEL POPULATION

Two third of the *Shaker* channel family members are expressed in guard cells. Besides AKT2, also KAT1, KAT2, AKT1, AtKC1, and GORK are detectable there and have important impacts on stomatal opening and closure (Szyroki et al., 2001; Ivashikina et al., 2005; Lebaudy et al., 2008b). Vast signal transduction pathways coordinate stomatal movement. In case of stomatal opening, they result in the activation of K_{in} channels and the uptake of $K^+$and anions, which finally leads to an increase of guard cell turgor. In case of stomatal closure, K_{out} channels are activated, K^+ is released together with anions, water is passively flowing out and guard cell turgor decreases. Recent comprehensive reviews of signal transduction pathways that affect stomatal opening and closure were published by Pandey et al. (2007) or Kim et al. (2010). **Figure 3** shows an overview of the regulation of K^+ channels involved in stomatal movements.

Channel variability in guard cells

Although KAT1 represents the dominant K_{in} channel in guard cells, it is not essential for stomatal opening (Ichida et al., 1997; Kwak et al., 2001; Szyroki et al., 2001). The coevally expressed

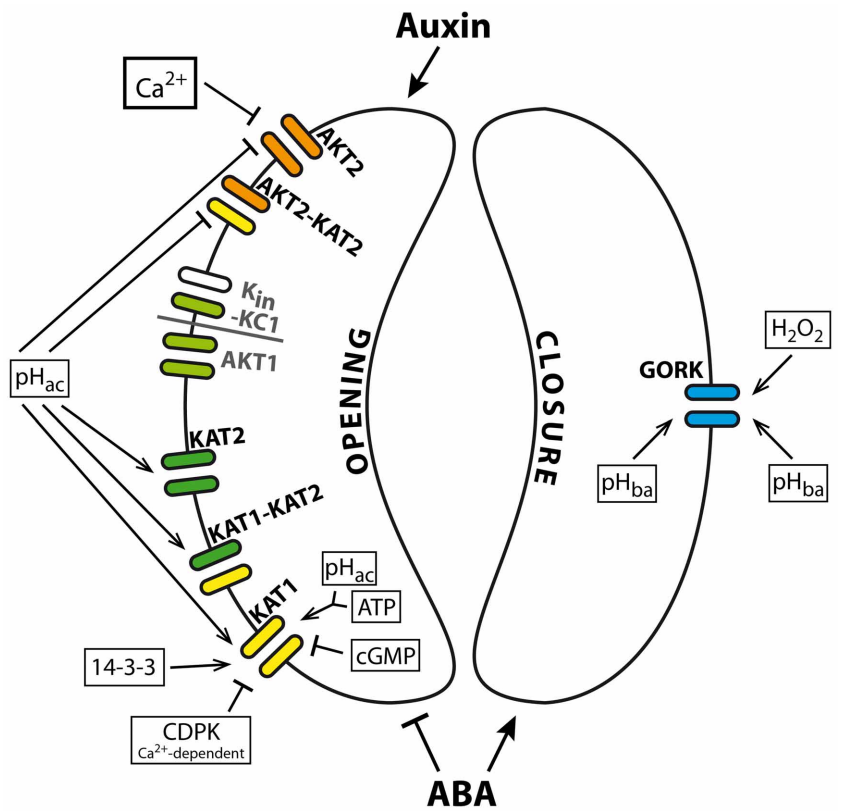

FIGURE 3 | K^+ channels in *Arabidopsis* guard cells and their effectors. Changes in membrane potential lead to stomatal opening or closure, respectively. Membrane hyperpolarisation in response to H^+-ATPase activity activates inward-rectifying K^+ channels. Transcripts for KAT1, KAT2, AKT1, AKT2, and AtKC1 are detectable in guard cells (Szyroki et al., 2001). All these K^+ channel subunits form heterotetrameric channels like KAT1-KAT2, AKT2-KAT2, and AKT1-AtKC1. The impact of AKT1 and K_{in}-AtKC1 on stomatal movement has not been investigated in detail. Acidification affects directly the currents through K^+ channels in guard cells. K_{in} channels are activated upon extracellular acidification, while currents through K_{weak} channels decrease. Besides, the K_{weak} channel AKT2 is negatively affected by extracellular Ca^{2+}. KAT1 currents are furthermore modulated by intracellular pH, ATP, and cGMP. ATP and cGMP have antagonistic effects. Moreover, stomatal K^+ channels are affected by signals via signal transduction cascades. Effects of 14-3-3 proteins, Ca^{2+} and kinases have been reported. Membrane depolarization, on the other hand, caused by the inactivation of H^+-ATPases and activation of anion channels activates the K_{out} channel GORK. GORK currents are positively influenced by extra- and intra-cellular alkalinisation. Furthermore, the current enhancing effect of H_2O_2 is under investigation. Both, stomatal opening and closure are affected by phytohormones. While Auxin evokes stomatal opening, ABA inhibits its opening but evokes closure of stomata. Abbreviations: pH_{ac}, acidification; pH_{ba}, alkalinisation.

K_{in} channel subunits AKT1, AKT2, and KAT2 are able to compensate for the loss of KAT1. The expression pattern of all guard cell K_{in} channel subunits is not exactly identical as exemplified by KAT1 and KAT2. Both subunits are expressed in guard cells. But, KAT1 is only expressed in guard cells of leaves and petioles, while KAT2 is additionally expressed in guard cells of the stem (Nakamura et al., 1995; Pilot et al., 2001). That points to different available sets of K^+ channels dependent on the guard cell location. Furthermore, K^+ channel subunits are able to form heteromeric channels in plants (Dreyer et al., 1997; Lebaudy et al., 2008a). For KAT1-KAT2 heterotetramers it has been shown that their basic properties are similar to properties observed for the homotetrameric KAT1 and KAT2 channels (Pilot et al., 2001; Lebaudy et al., 2010). In contrast, the AKT2-KAT2 heterotetramer combines different properties of its parental channels and forms a new functional type of a K^+ channel (Xicluna et al., 2007). The gating properties of the heterotetramer are inherited from AKT2, a weak-rectifying K^+ channel described above. The sensitivities to Ca^{2+} and H^+ are inherited from KAT2. Thus, K^+ channel heteromers notably contribute to an increase in channel variability and enhance the regulatory possibilities of K^+ channels.

K_{in} channels contribute to stomatal opening

For activation of K_{in} channels the membrane potential needs to be hyperpolarized. Hyperpolarization is achieved through the activity of H^+-ATPases that transport protons under ATP consumption out of the cell. The membrane voltage is sensed by the intrinsic voltage sensor that is formed by the transmembrane regions S1–S4. An important role is played especially by the positive charges in S4 (**Figure 1A**). The four voltage sensors of the channel induce conformational changes in the protein that then result in an opening of the permeation pathway. This voltage-sensitivity is modulated by other factors that interact with the channel protein. Indeed, many experiments show the sensitivity of guard cell K_{in} channels to changes in pH (Hedrich et al., 1995; Hoshi, 1995; Marten et al., 1999; Pilot et al., 2001; Xicluna et al., 2007). KAT1, KAT2 and the heteromeric KAT1-KAT2 are activated by extracellular and intracellular acidification due to a shift of the voltage dependence of the channels to more positive values. A histidine residue conserved among plant K_{in} channels that is located in the pore was suggested to sense pH changes in K_{in} channels (Hoth et al., 1997; Hoth and Hedrich, 1999). For KST1, a K_{in} channel from potato guard cells, it has been shown that this histidine is part of the pH sensor. Surprisingly, mutations of this histidine in KAT1 did not affect its pH dependence. Further investigation revealed that KAT1 senses pH changes via a sensory cloud rather than a single residue (Gonzalez et al., 2012). Besides, KAT1 is also modulated by ATP and cGMP. While cGMP reduces KAT1 currents, ATP affects KAT1 positively. Thus, ATP and cGMP show antagonistic effects (Hoshi, 1995).

Another regulator of guard cell K_{in} currents might be extracellular Ca^{2+}. Here, AKT2 is the only channel affected directly by external Ca^{2+} (Marten et al., 1999; Latz et al., 2007b). While AKT2 is blocked by Ca^{2+}, KAT1, KAT2 and AKT1 do not show any response (Szyroki et al., 2001; Ivashikina et al., 2005; Brüggemann et al., 1999). It is therefore proposed that experimentally observed sensitivity of guard cell K_{in} channels to extracellular Ca^{2+} is conferred by AKT2 subunits (Ivashikina et al., 2005).

Furthermore, effects of regulatory proteins on KAT1 have been shown. For instance, KAT1 is phosphorylated in a Ca^{2+}-dependent manner in the presence of CDPK-a Ca^{2+}-dependent protein kinase with a calmodulin-like domain (Li et al., 1998). This study used a recombinant CDPK from the bean *Vicia faba* and did not show whether KAT1 is phosphorylated directly by CDPK or rather other proteins are affected by the kinase. Berkowitz et al. (2000) showed in electrophysiological experiments that the recombinant CDPK has a negative effect on KAT1 currents. Ca^{2+}-dependent phosphorylation of KAT1 is further supported by a study that manipulated a protein kinase C (PKC) present in *X. laevis* oocytes (Sato et al., 2010). Upon activation of PKC that has similar target sites as plant Ca^{2+}-dependent kinases, KAT1 currents decline. In addition, recombinant 14-3-3 proteins from maize stimulated KAT1 currents (Sottocornola et al., 2006, 2008). These studies provide a first glimpse on the broad range of feasible effectors of K^+ channels in guard cells.

Additionally it was found that the channel population within the membrane undergoes regulation as well (Mikosch et al., 2006, 2009; Sutter et al., 2006, 2007; Sieben et al., 2008; Reuff et al., 2010). It has been shown that KAT1 interacts with SNARE proteins (see above), and ABA triggers endocytosis of KAT1 from the plasma membrane. Furthermore, the ER export motif of KAT1 subunits is important for proper channel trafficking. It has been shown that efficient ER export of KAT1 depends on an acidic motif in the C-terminus. Therefore, endo- and exocytosis, as well as the ER export of K^+ channels might be another level for regulating channel densities and K^+ currents across the membrane.

K_{out} channels during stomatal closure

K_{out} channels are activated upon depolarization. Such a membrane voltage change is achieved by inhibition of the H^+-ATPase and activation of anion channels. GORK is the only K_{out} channel identified in guard cells and is responsible for stomatal closure (Szyroki et al., 2001; Hosy et al., 2003). In contrast to inward rectifying channels, GORK currents are reduced with decreasing internal and external pH (Blatt, 1992; Ache et al., 2000). GORK also senses the external K^+ concentration, so that at higher external K^+ it requires more positive voltage for its activation (Ache et al., 2000). A similar analogy to SKOR might also hold for the direct interaction of GORK with the stress signaling molecule H_2O_2. GORK and SKOR share the cysteine residue that has been shown to be responsible for the activation effect in SKOR (Garcia-Mata et al., 2010). Nevertheless, the impact of H_2O_2 on GORK and the role of this presumed regulation in guard cell physiology still need to be investigated. Earlier reports have shown that H_2O_2 is an important player in stomatal signaling (reviewed by Wang and Song, 2008).

Alongside the activation of K_{out} channels during stomatal closure, K_{in} channels are deactivated (Blatt, 1990; Thiel et al., 1992). A knock-out mutant of the K_{in} channel AKT1 has been shown to be more resistant toward water stress than wild type plants (Nieves-Cordones et al., 2012). Transpiration was reduced and stomata closure was more efficient in knock-out plants treated with ABA. Thus, the inactivation of K_{in} channels favors stomatal

closure but it is not essential for the process of closure itself (MacRobbie, 1998). Furthermore, very similar phenotypes of *akt1* and *cipk23* knock-out plants could be observed suggesting a regulation of AKT1 by CIPK23 also in guard cells as it has been shown already for AKT1 in roots.

Influences of phytohormones

The phytohormones auxin and ABA cause opposing effects on stomata. Auxin is involved in plant developmental processes and promotes stomatal opening, whereas, ABA is involved in various stress responses. It prevents the opening and promotes the closure of stomata (Gehring et al., 1990). The direct influence of ABA on guard cell K^+ currents was shown by Blatt and Armstrong (1993). ABA treatment leads to inactivation of K_{in} and activation of K_{out} channels in guard cells. Although the phytohormon affects the transcription level of the K_{out} channel GORK in roots and shoots, the transcript level in guard cells remains unaffected (Becker et al., 2003). Besides, electrophysiological analyses exclude the direct effect of ABA on outward currents in guard cells. Therefore, ABA seems to affect guard cell K_{out} currents indirectly. As ABA signals from roots come along with alkalinisation of the guard cell cytoplasm (Blatt and Armstrong, 1993), the pH sensitive GORK can be activated and affected by ABA by this long distance signaling pathway (Blatt, 1992; Ache et al., 2000; Becker et al., 2003).

Auxin, on the other hand, stimulates the transcription of *KAT1* and *KAT2* (Philippar et al., 2004). It is not clear, however, whether this stimulation is tissue specific as in the case of GORK or whether it is a general feature of these K_{in} channel genes in different parts of the plant.

INFLUENCE ON POLLEN TUBE DEVELOPMENT

The *Shaker* channel SPIK is the main K_{in} channel in pollen and exclusively expressed there (Mouline et al., 2002; Zhao et al., 2013). Its disruption affects negatively pollen tube growth. The activity of SPIK is enhanced by decreasing external pH and negatively affected by the Ca^{2+}-dependent protein kinases CDPK11 and CDPK24. Ca^{2+} affects K_{in} currents only in the pollen tube but not in pollen grain protoplasts. It has been shown that the effect of Ca^{2+} is dependent on the presence of both kinases. In the absence of one of the kinases Ca^{2+} cannot block pollen K_{in} currents. Zhao and colleagues propose that Ca^{2+} acts negatively on SPIK via a kinase cascade, in which CDPK11 phosphorylates CDPK24.

CONCLUSIONS

K^+ channels are important for K^+ uptake from the soil, its distribution within the plant and processes to maintain and support plant growth. The past two decades revealed crucial information especially for plant *Shaker* like channels regarding the structure, physiological role and-to a minor extent-regarding their regulation. In contrast, our knowledge on TPK channels is far more rudimentary. The challenge of the future of plant K^+ channel research will be to identify the complex regulatory networks that regulate their activity and to understand the dynamics of these networks.

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REFERENCES

Ache, P., Becker, D., Ivashikina, N., Dietrich, P., Roelfsema, M., and Hedrich, R. (2000). GORK, a delayed outward rectifier expressed in guard cells of *Arabidopsis thaliana*, is a K^+-selective, K^+-sensing ion channel. *FEBS Lett.* 486, 93–98. doi: 10.1016/S0014-5793(00)02248-1

Alemán, F., Nieves-Cordones, M., Martínez, V., and Rubio, F. (2011). Root K^+ acquisition in plants: the *Arabidopsis thaliana* model. *Plant Cell Physiol.* 52, 1603–1612. doi: 10.1093/pcp/pcr096

Amtmann, A., Armengaud, P., and Volkov, V. (2004). "Potassium nutrition and salt stress," in *Membrane Transport in Plants*, ed M. R. Blatt (Oxford: Blackwell), 316–348.

Amtmann, A., Troufflard, S., and Armengaud, P. (2008). The effect of potassium nutrition on pest and disease resistance in plants. *Physiol. Plant.* 133, 682–691. doi: 10.1111/j.1399-3054.2008.01075.x

Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., and Gaber, R. F. (1992). Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3736–3740.

Basset, M., Conejero, G., Lepetit, M., Fourcroy, P., and Sentenac, H. (1995). Organization and expression of the gene coding for the potassium transport system AKT1 of *Arabidopsis thaliana*. *Plant Mol. Biol.* 29, 947–958.

Batistic, O., and Kudla, J. (2004). Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta* 219, 915–924. doi: 10.1007/s00425-004-1333-3

Becker, D., Geiger, D., Dunkel, M., and Roller, A. (2004). AtTPK4, an *Arabidopsis* tandem-pore K^+ channel, poised to control the pollen membrane voltage in a pH- and Ca^{2+}-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15621–15626.

Becker, D., Hoth, S., Ache, P., Wenkel, S., Roelfsema, M. R. G., Meyerhoff, O., et al. (2003). Regulation of the ABA-sensitive *Arabidopsis* potassium channel gene GORK in response to water stress. *FEBS Lett.* 554, 119–126. doi: 10.1016/S0014-5793(03)01118-9

Berkowitz, G., Zhang, X., Mercier, R., Leng, Q., and Lawton, M. (2000). Co- expression of calcium-dependent protein kinase with the inward rectified guard cell K^+ channel KAT1 alters current parameters in *Xenopus laevis* oocytes. *Plant Cell Physiol.* 41, 785–790. doi: 10.1093/pcp/41.6.785

Bihler, H., Eing, C., Hebeisen, S., Roller, A., Czempinski, K., and Bertl, A. (2005). TPK1 is a vacuolar ion channel different from the slow-vacuolar cation channel. *Plant Physiol.* 139, 417–424. doi: 10.1104/pp.105.065599

Blatt, M. R. (1990). Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. *Planta* 180, 445–455. doi: 10.1007/BF01160403

Blatt, M. R. (1992). K^+ channels of stomatal guard cells. Characteristics of the inward rectifier and its control by pH. *J. Gen. Physiol.* 99, 615–644.

Blatt, M. R., and Armstrong, F. (1993). K^+ channels of stomatal guard cells: abscisic- acid-evoked control of the outward rectifier mediated by cytoplasmic pH. *Planta* 191, 330–341. doi: 10.1007/BF00195690

Brüggemann, L., Dietrich, P., Dreyer, I., and Hedrich, R. (1999). Pronounced differences between the native K^+ channels and KAT1 and KST1 alpha-subunit homomers of guard cells. *Planta* 207, 370–376. doi: 10.1007/s004250050494

Caballero, F., Botella, M. A., Rubio, L., Fernández, J. A., Martínez, V., and Rubio, F. (2012). A Ca^{2+}-sensitive system mediates low-affinity K^+ uptake in the absence of AKT1

in *Arabidopsis* plants. *Plant Cell Physiol.* 53, 2047–2059. doi: 10.1093/pcp/pcs140

Càrdenas, L., Holdaway-Clarke, T. L., Saìnchez, F., Quinto, C., Feijoì, J. A., Kunkel, J. G., et al. (2000). Ion changes in legume root hairs responding to Nod factors. *Plant Physiol.* 123, 443–452. doi: 10.1104/pp.123.2.443

Chèrel, I., Michard, E., Platet, N., Mouline, K., Alcon, C., Sentenac, H., et al. (2002). Physical and functional interaction of the *Arabidopsis* K^+ channel AKT2 and phosphatase AtPP2CA. *Plant Cell* 14, 1133–1146. doi: 10.1105/tpc.000943

Chanroj, S., Wang, G., Venema, K., Zhang, M. W., Delwiche, C. F., and Sze, H. (2012). Conserved and diversified gene families of monovalent cation/H^+ antiporters from algae to flowering plants. *Front. Plant Sci.* 3:25. doi: 10.3389/fpls.2012.00025

Czempinski, K., Frachisse, J.-M., Maurel, C., Barbier-Brygoo, H., and Mueller-Roeber, B. (2002). Vacuolar membrane localization of the *Arabidopsis* 'two- pore' K^+ channel KCO1. *Plant J.* 29, 809–820. doi: 10.1046/j.1365-313X.2002.01260.x

Czempinski, K., Gaedeke, N., Zimmermann, S., and Mulller-Rolber, B. (1999). Molecular mechanisms and regulation of plant ion channels. *J. Exp. Bot.* 50, 955–966.

Czempinski, K., Zimmermann, S., Ehrhardt, T., and Mulller-Rolber, B. (1997). New structure and function in plant K^+ channels: KCO1, an outward rectifier with a steep Ca^{2+} dependency. *EMBO J.* 16, 2565–2575. doi: 10.1093/emboj/16.10.2565

Deeken, R., Geiger, D., Fromm, J., Koroleva, O., Ache, P., Langenfeld-Heyser, R., et al. (2002). Loss of the AKT2/3 potassium channel affects sugar loading into the phloem of *Arabidopsis*. *Planta* 216, 334–344. doi: 10.1007/s00425-002-0895-1

Deeken, R., Ivashikina, N., Czirjak, T., Philippar, K., Becker, D., Ache, P., et al. (2003). Tumour development in *Arabidopsis thaliana* involves the *Shaker*-like K^+ channels AKT1 and AKT2/3. *Plant J.* 34, 778–787. doi: 10.1046/j.1365-313X.2003.01766.x

Deeken, R., Sanders, C., Ache, P., and Hedrich, R. (2000). Developmental and light-dependent regulation of a phloem-localised K^+ channel of *Arabidopsis thaliana*. *Plant J.* 23, 285–290. doi: 10.1046/j.1365-313x.2000.00791.x

Dennison, K. L., Robertson, W. R., Lewis, B. D., Hirsch, R. E., Sussman, M. R., and Spalding, E. P. (2001). Functions of AKT1 and AKT2 potassium channels determined by studies of single and double mutants of *Arabidopsis*. *Plant Physiol.* 127, 1012–1019. doi: 10.1104/pp.010193

Dreyer, I., Antunes, S., Hoshi, T., Mulller-Rolber, B., Palme, K., Pongs, O., et al. (1997). Plant K^+ channel alpha-subunits assemble indiscriminately. *Biophys. J.* 72, 2143–2150.

Dreyer, I., and Blatt, M. R. (2009). What makes a gate? The ins and outs of Kv-like K^+ channels in plants. *Trends Plant Sci.* 14, 383–390. doi: 10.1016/j.tplants.2009.04.001

Dreyer, I., Horeau, C., Lemaillet, G., Zimmermann, S., Bush, D. R., Rodríguez-Navarro, A., et al. (1999). Identification and characterization of plant transporters. *J. Exp. Bot.*50, 1073–1087.

Dreyer, I., Michard, E., Lacombe, B., and Thibaud, J. B. (2001). A plant *Shaker*-like K^+ channel switches between two distinct gating modes resulting in either inward-rectifying or "leak" current. *FEBS Lett.* 505, 233–239. doi: 10.1016/S0014-5793(01)02832-0

Dreyer, I., Poree, F., Schneider, A., Mittelstadt, J., Bertl, A., Sentenac, H., et al. (2004). Assembly of plant *Shaker*-like K_{out} channels requires two distinct sites of the channel alpha-subunit. *Biophys. J.* 87, 858–872.

Duby, G., Hosy, E., Fizames, C., Alcon, C., Costa, A., Sentenac, H., et al. (2008). *At*KC1, a conditionally targeted *Shaker*-type subunit, regulates the activity of plant K^+ channels. *Plant J.* 53, 115–123. doi: 10.1111/j.1365-313X.2007.03324.x

Dunkel, M., Latz, A., Schumacher, K., Mulller, T., Becker, D., and Hedrich, R. (2008). Targeting of vacuolar membrane localized members of the TPK channel family. *Mol. Plant* 1, 938–949.

Ehrhardt, T., Zimmermann, S., and Müller-Röber, B. (1997). Association of plant K^+_{in} channels is mediated by conserved C-termini and does not affect subunit assembly. *FEBS Lett.* 409, 166–170. doi: 10.1016/S0014-5793(97)00502-4

Gajdanowicz, P., Michard, E., Sandmann, M., Rocha, M., Correâ, L. G. G., Ramìrez-Aguilar, S. J., et al. (2011). Potassium (K^+) gradients serve as a mobile energy source in plant vascular tissues. *Proc. Natl. Acad. Sci. U.S.A.* 108, 864–869.

Gapper, C., and Dolan, L. (2006). Control of plant development by reactive oxygen species. *Plant Physiol.* 141, 341–345. doi: 10.1104/pp.106.079079

Garcia-Mata, C., Wang, J., Gajdanowicz, P., Gonzalez, W., Hills, A., Donald, N., et al. (2010). A minimal cysteine motif required to activate the SKOR K^+ channel of *Arabidopsis* by the reactive oxygen species H_2O_2. *J. Biol. Chem.* 285, 29286–29294.

Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., et al. (1998). Identification and disruption of a plant *Shaker*-like outward channel involved in K^+ release into the xylem sap. *Cell* 94, 647–655. doi: 10.1016/S0092-8674(00)81606-2

Gehring, C. A., Irving, H. R., and Parish, R. W. (1990). Effects of auxin and abscisic acid on cytosolic calcium and pH in plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9645–9649.

Geiger, D., Becker, D., Vosloh, D., Gambale, F., Palme, K., Rehers, M., et al. (2009). Heteromeric *At*KC1-AKT1 channels in *Arabidopsis* roots facilitate growth under K^+-limiting conditions. *J. Biol. Chem.* 284, 21288–21295.

Gierth, M., Maser, P., and Schroeder, J. I. (2005). The potassium transporter *At*HAK5 functions in K^+ deprivation-induced high-affinity K^+ uptake and AKT1 K^+ channel contribution to K^+ uptake kinetics in *Arabidopsis* roots. *Plant Physiol.* 137, 1105–1114. doi: 10.1104/pp.104.057216

Gierth, M., and Mäser, P. (2007). Potassium transporters in plants–involvement in K^+ acquisition, redistribution and homeostasis. *FEBS Lett.* 581, 2348–2356. doi: 10.1016/j.febslet.2007.03.035

Gobert, A., Isayenkov, S., Voelker, C., Czempinski, K., and Maathuis, F. J. M. (2007). The two-pore channel TPK1 gene encodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10726–10731.

Gomez-Porras, J. L., Riaño-Pachón, D. M., Benito, B., Haro, R., Sklodowski, K., Rodriguez-Navarro, A., et al. (2012). Phylogenetic analysis of K^+ transporters in bryophytes, lycophytes, and flowering plants indicates a specialization of vascular plants. *Front. Plant Sci.* 3:167. doi: 10.3389/fpls.2012.00167

Gonzalez, W., Riedelsberger, J., Morales-Navarro, S. E., Caballero, J., Alzate-Morales, J. H., Gonzaìlez-Nilo, F. D., et al. (2012). The pH-sensor of the plant K^+ uptake channel KAT1 is built of a sensory cloud rather than of single key amino acids. *Biochem. J.* 442, 57–63.

Grefen, C., and Blatt, M. R. (2012). Do calcineurin B-like proteins interact independently of the serine threonine kinase CIPK23 with the K^+ channel AKT1? Lessons learned from a meìnage aì trois. *Plant Physiol.* 159, 915–919. doi: 10.1104/pp.112.198051

Grefen, C., Chen, Z., Honsbein, A., Donald, N., Hills, A., and Blatt, M. R. (2010). A novel motif essential for SNARE interaction with the K^+ channel KC1 and channel gating in *Arabidopsis*. *Plant Cell* 22, 3076–3092. doi: 10.1105/tpc.110.077768

Hamamoto, S., Marui, J., Matsuoka, K., Higashi, K., Igarashi, K., Nakagawa, T., et al. (2008a). Characterization of a tobacco TPK-type K^+ channel as a novel tonoplast K^+ channel using yeast tonoplasts. *J. Biol. Chem.* 283, 1911–1920.

Hamamoto, S., Yabe, I., and Uozumi, N. (2008b). Electrophysiological properties of NtTPK1 expressed in yeast tonoplast. *Biosci. Biotechnol. Biochem.* 72, 2785–2787.

Hedrich, R., Moran, O., Conti, F., Busch, H., Becker, D., Gambale, F., et al. (1995). Inward rectifier potassium channels in plants differ from their animal counterparts in response to voltage and channel modulators. *Eur. Biophys. J.* 24, 107–115.

Held, K., Pascaud, F., Eckert, C., Gajdanowicz, P., Hashimoto, K., Corratgeì-Faillie, C., et al. (2011). Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. *Cell Res.* 21, 1116–1130. doi: 10.1038/cr.2011.50

Hirsch, R. E., Lewis, B. D., Spalding, E. P., and Sussman, M. R. (1998). A role for the AKT1 potassium channel in plant nutrition. *Science* 280, 918–921. doi: 10.1126/science.280.5365.918

Honsbein, A., Sokolovski, S., Grefen, C., Campanoni, P., Pratelli, R., Paneque, M., Chen, Z., et al. (2009). A tripartite SNARE-K^+ channel complex mediates in channel-dependent K^+ nutrition in *Arabidopsis*. *Plant Cell* 21, 2859–2877. doi: 10.1105/tpc.109.066118

Hoshi, T. (1995). Regulation of voltage dependence of the KAT1 channel by intracellular factors. *J. Gen. Physiol.* 105, 309–328.

Hosy, E., Vavasseur, A., Mouline, K., Dreyer, I., Gaymard, F., Poree,

F., et al. (2003). The *Arabidopsis* outward K^+ channel GORK is involved in regulation of stomatal movements and plant transpiration. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5549–5554.

Hoth, S., Dreyer, I., Dietrich, P., Becker, D., Müller-Röber, B., and Hedrich, R. (1997). Molecular basis of plant-specific acid activation of K^+ uptake channels. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4806–4810.

Hoth, S., and Hedrich, R. (1999). Distinct molecular bases for pH sensitivity of the guard cell K^+ channels KST1 and KAT1. *J. Biol. Chem.* 274, 11599–11603.

Ichida, A. M., Pei, Z. M., Baizabal-Aguirre, V. M., Turner, K. J., and Schroeder, J. I. (1997). Expression of a Cs^+-resistant guard cell K^+ channel confers Cs^+- resistant, light-induced stomatal opening in transgenic *Arabidopsis*. *Plant Cell* 9, 1843–1857.

Isayenkov, S., Isner, J. C., and Maathuis, F. J. (2011a). Rice two-pore K^+ channels are expressed in different types of vacuoles. *Plant Cell* 23, 756–768.

Isayenkov, S., Isner, J. C., and Maathuis, F. J. (2011b). Membrane localisation diversity of TPK channels and their physiological role. *Plant Signal Behav.* 6, 1201–1204.

Isayenkov, S., and Maathuis, F. J. (2013). *Arabidopsis thaliana* vacuolar TPK channels form functional K^+ uptake pathways in *Escherichia coli*. *Plant Signal. Behav.* 8, e24665.

Ivashikina, N., Becker, D., Ache, P., Meyerhoff, O., Felle, H. H., and Hedrich, R. (2001). K^+ channel profile and electrical properties of *Arabidopsis* root hairs. *FEBS Lett.* 508, 463–469. doi: 10.1016/S0014-5793(01)03114-3

Ivashikina, N., Deeken, R., Fischer, S., Ache, P., and Hedrich, R. (2005). AKT2/3 subunits render guard cell K^+ channels Ca^{2+} sensitive. *J. Gen. Physiol.* 125, 483–492.

Jeanguenin, L., Alcon, C., Duby, G., Boeglin, M., Chèrel, I., Gaillard, I., et al. (2011). *At*KC1 is a general modulator of *Arabidopsis* inward *Shaker* channel activity. *Plant J.* 67, 570–582. doi: 10.1111/j.1365-313X.2011.04617.x

Johansson, I., Wulfetange, K., Poree, F., Michard, E., Gajdanowicz, P., Lacombe, B., et al. (2006). External K^+ modulates the activity of the *Arabidopsis* potassium channel SKOR via an unusual mechanism. *Plant J.* 46, 269–281. doi: 10.1111/j.1365-313X.2006.02690.x

Kim, T. H., Böhmer, M., Hu, H., Nishimura, N., and Schroeder, J. I. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. *Annu. Rev. Plant Biol.* 61, 561–591. doi: 10.1146/annurev-arplant-042809-112226

Kudla, J., Batistic, O., and Hashimoto, K. (2010). Calcium signals: the lead currency of plant information processing. *Plant Cell* 22, 541–563. doi: 10.1105/tpc.109.072686

Kwak, J. M., Murata, Y., Baizabal-Aguirre, V. M., Merrill, J., Wang, M., Kemper, A., et al. (2001). Dominant negative guard cell K^+ channel mutants reduce inward-rectifying K^+ currents and light-induced stomatal opening in *Arabidopsis*. *Plant Physiol.* 127, 473–485. doi: 10.1104/pp.010428

Lacombe, B., Pilot, G., Gaymard, F., Sentenac, H., and Thibaud, J. B. (2000a). PH control of the plant outwardly-rectifying potassium channel SKOR. *FEBS Lett.* 466, 351–354.

Lacombe, B., Pilot, G., Michard, E., Gaymard, F., Sentenac, H., and Thibaud, J. B. (2000b). A *Shaker*-like K^+ channel with weak rectification is expressed in both source and sink phloem tissues of *Arabidopsis*. *Plant Cell* 12, 837–851.

Lagarde, D., Basset, M., Lepetit, M., Conejero, G., Gaymard, F., Astruc, S., et al. (1996). Tissue-specific expression of *Arabidopsis* AKT1 gene is consistent with a role in K^+ nutrition. *Plant J.* 9, 195–203. doi: 10.1046/j.1365-313X.1996.09020195.x

Lan, W.-Z., Lee, S.-C., Che, Y.-F., Jiang, Y.-Q., and Luan, S. (2011). Mechanistic analysis of AKT1 regulation by the CBL-CIPK-PP2CA interactions. *Mol. Plant* 4, 527–536.

Latz, A., Becker, D., Hekman, M., Muller, T., Beyhl, D., Marten, I., et al. (2007a). TPK1, a Ca^{2+}-regulated *Arabidopsis* vacuole two-pore K^+ channel is activated by 14–3-3 proteins. *Plant J.* 52, 449–459.

Latz, A., Ivashikina, N., Fischer, S., Ache, P., Sano, T., Becker, D., et al. (2007b). In planta AKT2 subunits constitute a pH- and Ca^{2+}- sensitive inward rectifying K^+ channel. *Planta* 225, 1179–1191.

Lebaudy, A., Hosy, E., Simonneau, T., Sentenac, H., Thibaud, J., and Dreyer, I. (2008a). Heteromeric K^+ channels in plants. *Plant J.* 54, 1076–1082.

Lebaudy, A., Vavasseur, A., Hosy, E., Dreyer, I., Leonhardt, N., Thibaud, J.-B., et al. (2008b). Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5271–5276.

Lebaudy, A., Pascaud, F., Veìry, A.-A., Alcon, C., Dreyer, I., Thibaud, J.-B., et al. (2010). Preferential KAT1-KAT2 heteromerization determines inward K^+ current properties in *Arabidopsis* guard cells. *J. Biol. Chem.* 285, 6265–6274.

Lebaudy, A., Véry, A., and Sentenac, H. (2007). K^+ channel activity in plants: genes, regulations and functions. *FEBS Lett.* 581, 2357–2366. doi: 10.1016/j.febslet.2007.03.058

Lee, S. C., Lan, W.-Z., Kim, B.-G., Li, L., Cheong, Y. H., Pandey, G. K., et al. (2007). A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15959–15964.

Leigh, R. A., and Wyn Jones, R. G. (1984). A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. *New Phytol.* 97, 1–13. doi: 10.1111/j.1469-8137.1984.tb04103.x

Li, J., Lee, Y. J., and Assmann, S. M. (1998). Guard cells possess a calcium- dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiol.* 116, 785–795. doi: 10.1104/pp.116.2.785

Li, L., Kim, B.-G., Cheong, Y. H., Pandey, G. K., and Luan, S. (2006). A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12625–12630.

Maathuis, F. J. M. (2009). Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* 12, 250–258.

Maathuis, F. J. M. (2011). Vacuolar two-pore K^+ channels act as vacuolar osmosensors. *New Phytol.* 191, 84–91. doi: 10.1111/j.1469-8137.2011.03664.x

MacRobbie, E. A. C. (1998). Signal transduction and ion channels in guard cells. *Philos. Trans. R. Soc. Lond. B.* 353, 1475–1488.

Maitrejean, M., Wudick, M. M., Voelker, C., Prinsi, B., Mueller-Roeber, B., Czempinski, K., et al. (2011). Assembly and sorting of the tonoplast potassium channel *At*TPK1 and its turnover by internalization into the vacuole. *Plant Physiol.* 156, 1783–1796. doi: 10.1104/pp.111.177816

Marcel, D., Müller, T., Hedrich, R., and Geiger, D. (2010). K+ transport characteristics of the plasma membrane tandem-pore channel TPK4 and pore chimeras with its vacuolar homologs. *FEBS letters* 584, 2433–2439. doi: 10.1016/j.febslet.2010.04.038

Marschner, H. (2012). *Mineral Nutrition of Higher Plants*. London: Academic Press.

Marten, I., Hoth, S., Deeken, R., Ache, P., Ketchum, K. A., Hoshi, T., et al. (1999). AKT3, a phloem-localized K^+ channel, is blocked by protons. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7581–7586.

Mäser, P., Thomine, S., Schroeder, J., Ward, J., Hirschi, K., Sze, H., et al. (2001). Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiol.* 126, 1646–1667. doi: 10.1104/pp.126.4.1646

Michard, E., Dreyer, I., Lacombe, B., Sentenac, H., and Thibaud, J.-B. (2005a). Inward rectification of the AKT2 channel abolished by voltage-dependent phosphorylation. *Plant J.* 44, 783–797.

Michard, E., Lacombe, B., Poreìe, F., Mueller-Roeber, B., Sentenac, H., Thibaud, J.-B., et al. (2005b). A unique voltage sensor sensitizes the potassium channel AKT2 to phosphoregulation. *J. Gen. Physiol.* 126, 605–617.

Mikosch, M., Hurst, A. C., Hertel, B., and Homann, U. (2006). Diacidic motif is required for efficient transport of the K^+ channel KAT1 to the plasma membrane. *Plant Physiol.* 142, 923–930. doi: 10.1104/pp.106.087064

Mikosch, M., Käberich, K., and Homann, U. (2009). ER export of KAT1 is correlated to the number of acidic residues within a triacidic motif. *Traffic* 10, 1481–1487. doi: 10.1111/j.1600-0854.2009.00962.x

Mouline, K., Veìry, A.-A., Gaymard, F., Boucherez, J., Pilot, G., Devic, M., et al. (2002). Pollen tube development and competitive ability are impaired by disruption of a *Shaker* K^+ channel in *Arabidopsis*. *Genes Dev.*16, 339–350. doi: 10.1101/gad.213902

Nakamura, R. L., McKendree, W. L., Hirsch, R. E., Sedbrook, J. C., Gaber, R. F., and Sussman, M. R. (1995). Expression of an *Arabidopsis* potassium channel gene in guard cells. *Plant Physiol.* 109, 371–374. doi: 10.1104/pp.109.2.371

Nieves-Cordones, M., Caballero, F., Martínez, V., and Rubio, F. (2012). Disruption of the *Arabidopsis thaliana* inward-rectifier K^+ channel AKT1 improves plant responses to water stress. *Plant Cell Physiol.* 53, 423–432. doi: 10.1093/pcp/pcr194

Pandey, S., Zhang, W., and Assmann, S. M. (2007). Roles of ion channels and transporters in guard cell signal transduction. *FEBS Lett.* 581, 2325–2336. doi: 10.1016/j.febslet.2007.04.008

Philippar, K., Büchsenschutz, K., Abshagen, M., Fuchs, I., Geiger, D., Lacombe, B., et al. (2003). The K^+ channel KZM1 mediates potassium uptake into the phloem and guard cells of the C4 grass *Zea mays*. *J. Biol. Chem.* 278, 16973–16981.

Philippar, K., Ivashikina, N., Ache, P., Christian, M., Lułthen, H., Palme, K., et al. (2004). Auxin activates KAT1 and KAT2, two K^+-channel genes expressed in seedlings of *Arabidopsis thaliana*. *Plant J.* 37, 815–827. doi: 10.1111/j.1365-313X.2003.02006.x

Pilot, G., Gaymard, F., Mouline, K., Chèrel, I., and Sentenac, H. (2003). Regulated expression of *Arabidopsis Shaker* K^+ channel genes involved in K^+ uptake and distribution in the plant. *Plant Mol. Biol.* 51, 773–787.

Pilot, G., Lacombe, B., Gaymard, F., Cherel, I., Boucherez, J., Thibaud, J. B., et al. (2001). Guard cell inward K^+ channel activity in *Arabidopsis* involves expression of the twin channel subunits KAT1 and KAT2. *J. Biol. Chem.* 276, 3215–3221.

Pyo, Y. J., Gierth, M., Schroeder, J. I., and Cho, M. H. (2010). High-affinity K^+ transport in *Arabidopsis*: *At*HAK5 and AKT1 are vital for seedling establishment and postgermination growth under low-potassium conditions. *Plant Physiol.* 153, 863–875. doi: 10.1104/pp.110.154369

Rajan, S., Preisig-Muller, R., Wischmeyer, E., Nehring, R., Hanley, P. J., Renigunta, V., et al. (2002). Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. *J. Physiol.* 545, 13–26. doi: 10.1113/jphysiol.2002.027052

Reintanz, B., Szyroki, A., Ivashikina, N., Ache, P., Godde, M., Becker, D., et al. (2002). *At*KC1, a silent *Arabidopsis* potassium channelalpha -subunit modulates root hair K^+ influx. *Proc. Natl. Acad. Sci. U.S.A.* 99, 4079–4084.

Ren, X.-L., Qi, G.-N., Feng, H.-Q., Zhao, S., Zhao, S.-S., Wang, Y., et al. (2013). Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K^+ homeostasis in *Arabidopsis*. *Plant J.* 74, 258–266. doi: 10.1111/tpj.12123

Reuff, M., Mikosch, M., and Homann, U. (2010). Trafficking, lateral mobility and segregation of the plant K^+ channel KAT1*. *Plant Biol.* 12, 99–104. doi: 10.1111/j.1438-8677.2010.00355.x

Rocchetti, A., Sharma, T., Wulfetange, C., Scholz-Starke, J., Grippa, A., Carpaneto, A., et al. (2012). The putative K^+ channel subunit *At*KCO3 forms stable dimers in *Arabidopsis*. *Front. Plant Sci.* 3:251. doi: 10.3389/fpls.2012.00251.

Rubio, F., Alemaìn, F., Nieves-Cordones, M., and Martiìnez, V. (2010). Studies on *ArabidopsisAt*HAK5, *At*AKT1 double mutants disclose the range of concentrations at which *At*HAK5, *At*AKT1 and unknown systems mediate K uptake. *Physiol. Plant.* 139, 220–228. doi: 10.1111/j.1399-3054.2010.01354.x

Rubio, F., Nieves-Cordones, M., Alemaìn, F., and Martiìnez, V. (2008). Relative contribution of *At*HAK5 and *At*AKT1 to K^+ uptake in the high-affinity range of concentrations. *Physiol. Plant* 134, 598–608. doi: 10.1111/j.1399-3054.2008.01168.x

Sandmann, M., Skłodowski, K., Gajdanowicz, P., Michard, E., Rocha, M., Gomez-Porras, J. L., et al. (2011). The K^+ battery-regulating *Arabidopsis* K^+ channel AKT2 is under the control of multiple post-translational steps. *Plant Signal. Behav.* 6, 558–562.

Sato, A., Gambale, F., Dreyer, I., and Uozumi, N. (2010). Modulation of the *Arabidopsis* KAT1 channel by an activator of protein kinase C in *Xenopus laevis* oocytes. *FEBS J.* 277, 2318–2328. doi: 10.1111/j.1742-4658.2010.07647.x

Schachtman, D. P., Schroeder, J. I., Lucas, W. J., Anderson, J. A., and Gaber, R. F. (1992). Expression of an inward-rectifying potassium channel by the *Arabidopsis* KAT1 cDNA. *Science* 258, 1654–1658. doi: 10.1126/science.8966547

Schönknecht, G., Spoormaker, P., Steinmeyer, R., Brüggemann, L., Ache, P., Dutta, R., et al. (2002). KCO1 is a component of the slow-vacuolar (SV) ion channel. *FEBS Lett.* 511, 28–32. doi: 10.1016/S0014-5793(01)03273-2

Schroeder, J. I., Ward, J. M., and Gassmann, W. (1994). Perspectives on the physiology and structure of inward-rectifying K^+channels in higher plants: biophysical implications for K^+ uptake. *Annu. Rev. Biophys. Biomol. Struc.* 23, 441–471.

Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J. M., Gaymard, F., et al. (1992). Cloning and expression in yeast of a plant potassium ion transport system. *Science* 256, 663–665. doi: 10.1126/science.1585180

Sieben, C., Mikosch, M., Brandizzi, F., and Homann, U. (2008). Interaction of the K^+-channel KAT1 with the coat protein complex II coat component Sec24 depends on a di-acidic endoplasmic reticulum export motif. *Plant J.* 56, 997–1006. doi: 10.1111/j.1365-313X.2008.03658.x

Sottocornola, B., Gazzarrini, S., Olivari, C., Romani, G., Valbuzzi, P., Thiel, G., et al. (2008). 14-3-3 proteins regulate the potassium channel KAT1 by dual modes. *Plant Biol.* 10, 231–236. doi: 10.1111/j.1438-8677.2007.00028.x

Sottocornola, B., Visconti, S., Orsi, S., Gazzarrini, S., Giacometti, S., Olivari, C., et al. (2006). The potassium channel KAT1 is activated by plant and animal 14-3-3 proteins. *J. Biol. Chem.* 281, 35735–35741.

Spalding, E. P., Hirsch, R. E., Lewis, D. R., Qi, Z., Sussman, M. R., and Lewis, B. D. (1999). Potassium uptake supporting plant growth in the absence of AKT1 channel activity: inhibition by ammonium and stimulation by sodium. *J. Gen. Physiol.* 113, 909–918.

Sutter, J.-U., Campanoni, P., Tyrrell, M., and Blatt, M. R. (2006). Selective mobility and sensitivity to SNAREs is exhibited by the *Arabidopsis* KAT1 K^+ channel at the plasma membrane. *Plant Cell* 18, 935–954. doi: 10.1105/tpc.105.038950

Sutter, J.-U., Sieben, C., Hartel, A., Eisenach, C., Thiel, G., and Blatt, M. R. (2007). Abscisic acid triggers the endocytosis of the *Arabidopsis* KAT1 K^+ channel and its recycling to the plasma membrane. *Curr. Biol.* 17, 1396–1402.

Szyroki, A., Ivashikina, N., Dietrich, P., Roelfsema, M. R., Ache, P., Reintanz, B., et al. (2001). KAT1 is not essential for stomatal opening. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2917–2921.

Thiel, G., MacRobbie, E. A. C., and Blatt, M. B. (1992). Membrane transport in stomatal guard cells: the importance of voltage control. *J. Membr. Biol.* 126, 1–18.

Torres, M. A., and Dangl, J. L. (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* 8, 397–403.

Tripathi, V., Parasuraman, B., Laxmi, A., and Chattopadhyay, D. (2009). CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. *Plant J.* 58, 778–790. doi: 10.1111/j.1365-313X.2009.03812.x

Véry, A., Gaymard, F., Bosseux, C., Sentenac, H., and Thibaud, J. (1995). Expression of a cloned plant K^+ channel in *Xenopus* oocytes: analysis of macroscopic currents. *Plant J.* 7, 321–332. doi: 10.1046/j.1365-313X.1995.7020321.x

Véry, A., and Sentenac, H. (2003). Molecular mechanisms and regulation of K^+ transport in higher plants. *Annu. Rev. Plant Biol.* 54, 575–603. doi: 10.1146/annurev.arplant.54.031902.134831

Vitale, A. and Hinz, G. (2005). Sorting of proteins to storage vacuoles: how many mechanisms? *Trends Plant Sci.* 10, 316–323.

Voelker, C., Gomez-Porras, J. L., Becker, D., Hamamoto, S., Uozumi, N., Gambale, F., et al. (2010). Roles of tandem-pore K^+ channels in plants - a puzzle still to be solved. *Plant Biol.* 12(Suppl. 1), 56–63. doi: 10.1111/j.1438-8677.2010.00353.x

Voelker, C., Schmidt, D., Mueller-Roeber, B., and Czempinski, K. (2006). Members of the *Arabidopsis* AtTPK/KCO family form homomeric vacuolar channels in planta. *Plant J.* 48, 296–306. doi: 10.1111/j.1365-313X.2006.02868.x

Wang, P., and Song, C. P. (2008). Guard-cell signalling for hydrogen peroxide and abscisic acid. *New Phytol.* 178, 703–718. doi: 10.1111/j.1469-8137.2008.02431.x

Wang, Y., and Wu, W.-H. (2010). Plant sensing and signaling in response to K^+- deficiency. *Mol. Plant* 3, 280–287.

Wyn Jones, R. G., and Pollard, A. (1983). "Proteins, enzymes and inorganic ions," in *Encyclopedia of Plant Physiology*, eds A. Lauchli and A. Pirson (Berlin: Springer), 528–562.

Xicluna, J., Lacombe, B., Dreyer, I., Alcon, C., Jeanguenin, L., Sentenac, H., et al. (2007). Increased functional diversity of plant K^+ channels by preferential heteromerization of the *Shaker*-like subunits AKT2 and KAT2. *J. Biol. Chem.* 282, 486–494.

Xu, J., Li, H.-D., Chen, L.-Q., Wang, Y., Liu, L.-L., He, L., et al. (2006). A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. *Cell* 125, 1347–1360. doi: 10.1016/j.cell.2006.06.011

Zanetti, M., Teardo, E., La Rocca, N., Zulkifli, L., Checchetto, V., Shijuku,

T., et al. (2010). A novel potassium channel in photosynthetic cyanobacteria. *PLoS ONE* 5:e10118. doi: 10.1371/journal.pone.0010118

Zhao, L.-N., Shen, L.-K., Zhang, W.-Z., Zhang, W., Wang, Y., and Wu, W.-H. (2013). Ca^{2+}-Dependent Protein Kinase11 and 24 Modulate the Activity of the Inward Rectifying K^+ Channels in Arabidopsis Pollen Tubes. *Plant Cell* 25, 649–661. doi: 10.1105/tpc.112.103184

Zhou, Y., Morais-Cabral, J. H., Kaufman, A., and MacKinnon, R. (2001). Chemistry of ion coordination and hydration revealed by a K^+ channel-Fab complex at 2.0 A resolution. *Nature* 414, 43–48. doi: 10.1038/35102009

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Are sucrose transporter expression profiles linked with patterns of biomass partitioning in *Sorghum* phenotypes?

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Sorghum bicolor is a genetically diverse C_4 monocotyledonous species, encompassing varieties capable of producing high grain yields as well as sweet types which accumulate soluble sugars (predominantly sucrose) within their stems to high concentrations. Sucrose produced in leaves (sources) enters the phloem and is transported to regions of growth and storage (sinks). It is likely that sucrose transporter (SUT) proteins play pivotal roles in phloem loading and the delivery of sucrose to growth and storage sinks in all *Sorghum* ecotypes. Six *SUTs* are present in the published *Sorghum* genome, based on the BTx623 grain cultivar. Homologues of these *SUTs* were cloned and sequenced from the sweet cultivar Rio, and compared with the publically available genome information. SbSUT5 possessed nine amino acid sequence differences between the two varieties. Two of the remaining five SUTs exhibited single variations in their amino acid sequences (SbSUT1 and SbSUT2) whilst the rest shared identical sequences. Complementation of a mutant *Saccharomyces* yeast strain (SEY6210), unable to grow upon sucrose as the sole carbon source, demonstrated that the *Sorghum* SUTs were capable of transporting sucrose. *SbSUT1*, *SbSUT4*, and *SbSUT6* were highly expressed in mature leaf tissues and hence may contribute to phloem loading. In contrast, *SbSUT2* and *SbSUT5* were expressed most strongly in sinks consistent with a possible role of facilitating sucrose import into stem storage pools and developing inflorescences.

Keywords: expression profiling, *Sorghum*, source–sink pathway, sucrose transporters, sucrose storage

INTRODUCTION

The storage of organic carbon as non-structural carbohydrates by plants is of biological and commercial interest. In this context, many varieties exist within the genetically diverse *Sorghum bicolor* species ranging from grain *Sorghum* types which store large amounts of starch within their grains to sweet *Sorghum* types which accumulate sucrose/hexoses within their stems. Sweet *Sorghum* cultivars are capable of accumulating soluble sugars up to 60% of their internode dry weight (Hoffmann-Thoma et al., 1996). The Rio cultivar can accumulate three times the amount of sugar (total glucose, fructose, and sucrose g/kg stem tissue) in mature stems compared to grain *Sorghum* cv. BTx623 (Murray et al., 2008). For these reasons, sweet *Sorghum*, a C_4 monocotyledonous plant with high yield potential, is regarded as an ideal feedstock to provide sugar for bioethanol production. Higher sugar lines are preferred for the production of "first generation" bioethanol. A high sugar variety may yield 500 g of sugar per kg of stem dry weight, and total soluble sugar yields can reach 10 t ha^{-1} (Zhao et al., 2009). These yields equate to theoretical ethanol yields of up to 5414 L ha^{-1} (Zhao et al., 2009). However, higher sugar, and hence ethanol yields per hectare may be achievable through selective breeding and/or genetic transformation of *Sorghum*.

During sugar accumulation within stems, sucrose produced in photosynthetic source leaves is transported within phloem sieve element-companion cell (SE-CC) complexes to an array of sinks (non-photosynthetic organs) comprising developing vegetative and reproductive organs (growth sinks) as well as the stem storage sink. Within growth sinks carbohydrates are invested primarily into the biosynthesis of cellular structures. In contrast, elongating and mature internodes of cv. Rio accumulate sucrose within vacuoles, cytosols, and apoplasmic spaces of their storage parenchyma cells (Lingle, 1987).

In the C_4 species maize (*Zea mays*), closely related to *Sorghum*, sucrose loading of SE-CC complexes occurs apoplasmically (Slewinski et al., 2009). It is assumed that a similar pathway of phloem loading of sucrose is followed in *Sorghum*. In stems of sugarcane and *Sorghum*, sucrose is transferred radially from their SE-CC complexes into storage parenchyma cells. Intracellular compartmentation of stored sucrose in *Sorghum* is presumed to be similar to that of sugarcane. Here, the bulk of sucrose accumulates within vacuoles of their storage parenchyma cells to concentrations that equal or exceed sucrose concentrations of the phloem sap. Thus the possibility of a concentrating step is invoked. Since the pathway of phloem unloading follows a symplasmic pathway in sugarcane stems (Jacobsen et al., 1992), any concentrating step must be localized to tonoplasts of their storage parenchyma vacuoles. Inconsistent with this conclusion is the finding that sucrose transport into isolated vacuoles of sugarcane stems occurs by facilitated diffusion (Williams et al., 1990; Preisser and Komor, 1991). However, whether an energy-dependent transport step operates in parallel with facilitated diffusion into vacuoles, as reported

for sugar beet (Saftner et al., 1983), remains to be resolved for sugarcane. In the case of *Sorghum*, the phloem unloading pathway of sucrose into stem storage parenchyma cells appears to include an apoplasmic component (Tarpley and Vietor, 2007) and hence an additional reliance on movement across plasma membranes arranged in series with tonoplast transport.

Import of sucrose into cells across their plasma membranes is mediated by sucrose transporters (SUTs). SUTs are energy-dependent trans-membrane proteins which co-transport sucrose and protons in the same direction, in a 1:1 stoichiometric ratio (Lalonde et al., 2004). Therefore, *Sorghum* SUTs are of interest because they may play key roles in apoplasmic phloem loading of sucrose in source leaves and apoplasmic unloading of sucrose into stem storage sinks (see above). SUTs are known to function in phloem loading of maize source leaves (Slewinski et al., 2009) but the role of SUTs in stem storage is less certain. Here the final sucrose concentration within stems can be a balance between import and remobilization to provide a supplementary source of organic carbon to support grain filling when leaf photosynthesis has been depressed by stressful conditions (Blum et al., 1994, 1997). However, remobilization of stem reserves in a number of *Sorghum* cultivars has been reported to be minimal under favorable environmental conditions (Gutjahr et al., 2013).

Here we investigate the expression of *Sorghum* SUTs in source and sink organs during vegetative growth and at anthesis in two cultivars of *Sorghum*, cv. BTx623 and cv. Rio. These two cultivars exhibit very different phenotypes, with cv. BTx623 being of short stature and producing a large grain head. In contrast, cv. Rio produces a small panicle with fewer grains, but may grow to a height of 3 m with a stout culm for sugar storage. Differences in *SUT* expression between cultivars may correlate with phloem loading, long distance transport, and ultimately partitioning of sucrose to reproductive sinks in cv. BTx623 or stem sinks in cv. Rio. Complementation of the deficient *Saccharomyces cerevisiae* SEY6210 strain by *Sorghum* SUTs is also explored as a first step toward detailed functional characterization of these transporters.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

Seeds of the *Sorghum* cultivars Rio and BTx623 were germinated and grown in 10 L pots containing a soil mixture consisting of two parts coarse sand, one part coco peat, and one part perlite, under glass house conditions with temperatures maintained at 25.5 ± 1.5°C during the day, and 15.5 ± 0.5°C during the night. Plants were exposed to a photoperiod of 14-h light and 10-h dark cycle with supplementary lighting provided by tungsten incandescent lamps. Seedlings were thinned to one per pot at 1-week post germination. Pot water levels were maintained at field capacity with a programmable drip irrigation system delivering water to each pot for two min, three times per day. Osmocote exact slow release fertilizer (Scotts Australia Pty Ltd, Sydney, NSW, Australia) was applied at a rate of 20 g per pot 2-weeks post germination and was supplemented with liquid fertilizer (Wuxal Liquid Foliar Nutrients; AgNova Technologies Pty Ltd, Eltham, VIC, Australia) at fortnightly intervals. Nitrogen (N), phosphorus (P), and potassium (K) ratios for Osmocote exact were 15N, 3.9P, and 9.1K.

HARVESTING PLANT MATERIAL

All plant samples were snap frozen in liquid nitrogen immediately following harvest. During the vegetative growth phase, cv. BTx623 (grain) and cv. Rio (sweet) were destructively harvested approximately 60 and 90 days after germination, respectively. Material harvested for analysis was a sink leaf (expanding leaf fully enclosed within leaf sheaths); source leaf (youngest fully expanded leaf), internode 2 (elongated internode; numbered acropetally), and internode 5 (elongating). At anthesis, cv. BTx623 and cv. Rio were harvested approximately 103 and 140 days after germination, respectively. The flag leaf and leaf 7 (numbered acropetally), the flag internode, internode 2 and whole inflorescences were harvested. Additional samples were taken for detailed analysis of SUT expression. These were upper portion (5 cm) of the flag internodes and inflorescences separated into spikelets, anthers, and rachis branches.

ISOLATION OF TOTAL RNA

Tissue samples were cryogenically ground in stainless steel grinding jars cooled on dry ice with a cooled stainless steel ball bearing agitated for 1 min at 30 Hz using a Retsch TissueLyser II (QIAGEN, Chadstone Centre, VIC, Australia). Total RNA was isolated from 100 mg of ground material. Leaves were extracted using the plant RNeasy® kit (QIAGEN) whilst stems and inflorescences were extracted using the plant RNA reagent (Life Technologies, Mulgrave, VIC, Australia). Digestion of contaminating genomic DNA was performed post RNA isolation using the Ambion® TURBO™ DNase kit (Life Technologies). RNA isolation and genomic DNA digestions were performed according to the manufacturer's instructions.

SYNTHESIS OF cDNA

Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the Thermoscript® first strand cDNA synthesis kit (Life technologies) with an oligo d(T) primer, at an extension temperature of 60°C, according to the manufacturer's instructions.

CLONING FULL-LENGTH GENES

Full-length coding DNA fragments of each *Sorghum SUT* was cloned from cDNA by polymerase chain reaction (PCR) using Fermentas 2xMM (ThermoFisher, Scoresby, VIC, Australia) spiked with 1 μL Fermentas Pfu polymerase (ThermoFisher) using gene specific primers (**Table 1**). PCR cycling conditions were 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min (2 min 20 s for *SbSUT2*). Amplified products were cloned into the pGEM-t easy vector (Promega, Sydney, NSW, Australia) and at least three clones were sequenced from separate cDNA samples. *SUTs* were then amplified from plasmids using the Stratagene Pfu Ultra II polymerase (Integrated Sciences, Chatswood, NSW, Australia) by primers incorporating restriction sites at the start and stop codons as shown in **Table 1** and recommended cycling profile using a 55°C annealing temperature. Products were digested with corresponding FastDigest® Fermentas restriction enzymes (ThermoFisher), as were the yeast expression vectors. *SUTs* were then ligated into pDR195 (*SbSUT5* and *SbSUT6*) or pDR196 (Rentsch et al., 1995).

Table 1 | Primer sets used for PCR amplification of *Sorghum SUT*s. Restriction site sequences are underlined.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Full-length primers		
SbSUT1	GTCGTCCCGTACGTGTGC	ATCTTGCACGGTTGGGTTT
SbSUT2	CCGCAGCGACACCTACAC	AATGGCAAAATGGGGCTAAGT
SbSUT3	CTCCACACCTCTCCGGTTT	CGACAGTAGTGGTTGATCG
SbSUT4	TCAAAGCAACTCAGCGATTC	AGCTGCAACTCTTCCAAAGC
SbSUT5	GTAGCCATGGACGGTGGTG	CCGCCTGGCGATAGATAGAT
SbSUT6	CGTTCCTGCTCCTCTCACTC	TGGATTTCCGATCATCCACT
Restriction cloning primers		
SbSUT1	CTCGCGGAATTCATGGCTCGCGGCGA EcoRI	GGCCGTGTCGACTCAGTGGCCGCCCG SalI
SbSUT2	GGCGCGGTCGACATGGACGCCGGCACC SalI	TTGGGCAGTCGACTCAGCCAAATCCATGG XhoI
SbSUT3	CCGGTTGAATTCATGGCTGCTGATGGC EcoRI	CTGGACCTCGAGTCAATGGCCTCCTC XhoI
SbSUT4	CCGTGAGAATTCATGCCGCCGCGCAC EcoRI	GTAATGAGTCGACTTATCGGTGCGTGC SalI
SbSUT5	AATTCGAGCGGCCGCATGGACGGTGGTGAC NotI	GCGATAGGATCCTCAGTGGCCGCCGC BamHI
SbSUT6	GCCCGGCGGCCGCATGGACGACGGTGAC NotI	CCTGGAGGATCCTCAACAGTGGCCGC BamHI
qPCR primers		
SbSUT1	GTGCTCCTGTAATCTTTGTGTCC	ACTATACTGCACATTGATTGATCG
SbSUT2	GCACATGCATTGAATGAACC	TTCGCATTTGGAAATTCCTC
SbSUT3	GGCCGGATCAAACAAGAT	GGCATTGCGAAGGAATGA
SbSUT4	CGATCCATGATGATGTCCAG	GTTCCAGGCCTTGCTGTC
SbSUT5	CCCGTAGTGTTGCGGAGTC	CCAATGGATCGGAAAATAAAG
SbSUT6	GCACAACAGCACAAAGAAGG	AGGCAGAAGAGGCTGAGATG
SbGAPDH	AGGGTATCATGGGCTACGTG	AGTTGTCGTTCAGGGCAATC
SbEF1a	CATGGTGGTGGAGACCTTCT	TCCTTCTTCTCCACGCTCTT

YEAST TRANSFORMATION

Sorghum SUT-yeast expression vector constructs were introduced into the *Saccharomyces cerevisiae* yeast strain SEY6210 (MATα leu2–3, 112 ura3–52 his3–Δ200 trpl-Δ901 lys2–801 suc2- Δ9 GAL; Robinson et al., 1988) using the 40% PEG1000 transformation method (Dohmen et al., 1991). Yeast transformants harboring one of each of the cv. Rio SUTs, the cv. BTx623 SbSUT5 (SbSUT5G) and empty pDR196 vector were identified. Media lacking uracil was used for selection as the pDR yeast expression vectors contain the uracil synthesis gene. DNA was extracted from yeast post transformation, then plasmids were transformed into *Escherichia coli* (strain DH5α), and were harvested using a Plasmid Mini Kit (QIAGEN). Plasmids were sequenced to confirm that the *SUT* sequences were correct. In short, 1.5 mL yeast culture was pelleted, washed with MilliQ water then resuspended in lysis buffer [50 mM Tris-HCl pH 8, 100 mM NaCl, 1% SDS, 2% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA)]. Glass beads were added (0.3 g, 425–600 μm diameter) along with 200 μL phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich, Castle Hill, NSW, Australia) and vortexed for 10 min followed by microcentrifugation for 5 min at maximum speed. The upper extract layer was then removed and DNA precipitated in 1 mL ethanol prior to pelleting and resuspension in 50 μL TE.

YEAST COMPLEMENTATION

Transformed yeast strains harboring *Sorghum* SUTs, empty pDR196 and PsSUT1 were grown in liquid culture to an OD_{600} of 0.8 in synthetic dropout media lacking uracil. Untransformed yeast was cultured in synthetic complete media. Yeast were streaked (2 μL) on solid media lacking uracil and supplemented with either sucrose (25 mM) or glucose (100 mM) as the sole carbon source. This was repeated three times and plates were photographed

using a ChemiDoc^{TM} XRS system (Bio-Rad, Gladesville, NSW, Australia). SuSy7 yeast harbouring PsSUT1-pDR196 was kindly provided by Zhou et al. (2007) for use as a positive control.

SUT TRANSCRIPT QUANTIFICATION BY qPCR

Primers used for quantitative PCR (qPCR; **Table 1**) were designed to amplify regions of the 3′ UTR of each *SUT* due to high sequence homology within coding regions, with the exception of *SbSUT2* where a region from the coding sequence was amplified. Products from standard PCR were sequenced to ensure that correct gene fragments were amplified. Quantitative PCR was carried out on a Rotor-Gene Q (QIAGEN) using the QuantiFast SYBR green PCR kit (QIAGEN) and a two-step cycling program according to the manufacturer's instructions. The green channel was used for data acquisition. Gene expression was measured relative to the housekeeper, *Sorghum bicolor elongation factor 1-alpha* (*SbEF-1α*).

SELECTION OF HOUSEKEEPING GENE FOR qPCR

The expression stability of two widely used housekeeping genes, *Sorghum bicolor glyceraldehyde-6-phosphate dehydrogenase* (*SbGAPDH*) and *SbEF-1α* from cv. Rio, were assessed prior to measuring expression levels of *Sorghum SUTs*. Comparison of cycle threshold values (Ct) and absolute expression levels (data not shown) revealed both housekeeping genes were quite stably expressed within each organ examined. However, differences in expression of *SbGAPDH* were greater than those for *SbEF-1α*. Hence *SbEF-1α* was chosen to normalize *SUT* expression in subsequent experiments. The stability of *SbEF-1α* was compared between cv. BTx623 and cv. Rio (**Figure 1**). Expression of *SbEF-1α* was least stable in cv. Rio during vegetative growth (Source leaf and Inter 2 – **Figure 1A**) and cv. BTx623 at anthesis (Inter 2 – **Figure 1E**). However, in all cases this variation was insignificant relative to the observed genotypic differences in the relative expression levels of the genes of interest and hence had no impact on the conclusions drawn.

RESULTS

SbSUT SEQUENCES

Full-length coding sequences of each *SUT* from both *Sorghum* cultivars were amplified by PCR, cloned, and then sequenced. Twelve trans-membrane domains were predicted for each SUT using the TMHMM (Hidden Markov model-based transmembrane) predictive algorithm, and a graphical representation of the membrane topology of SbSUT5 is shown (**Figure 2**). Cytoplasmic N- and C-termini were predicted along with a central loop domain. Sequence analysis (not shown) revealed that a number of conserved features are present in *Sorghum* SUTs. A conserved histidine residue is present in the first loop domain corresponding to

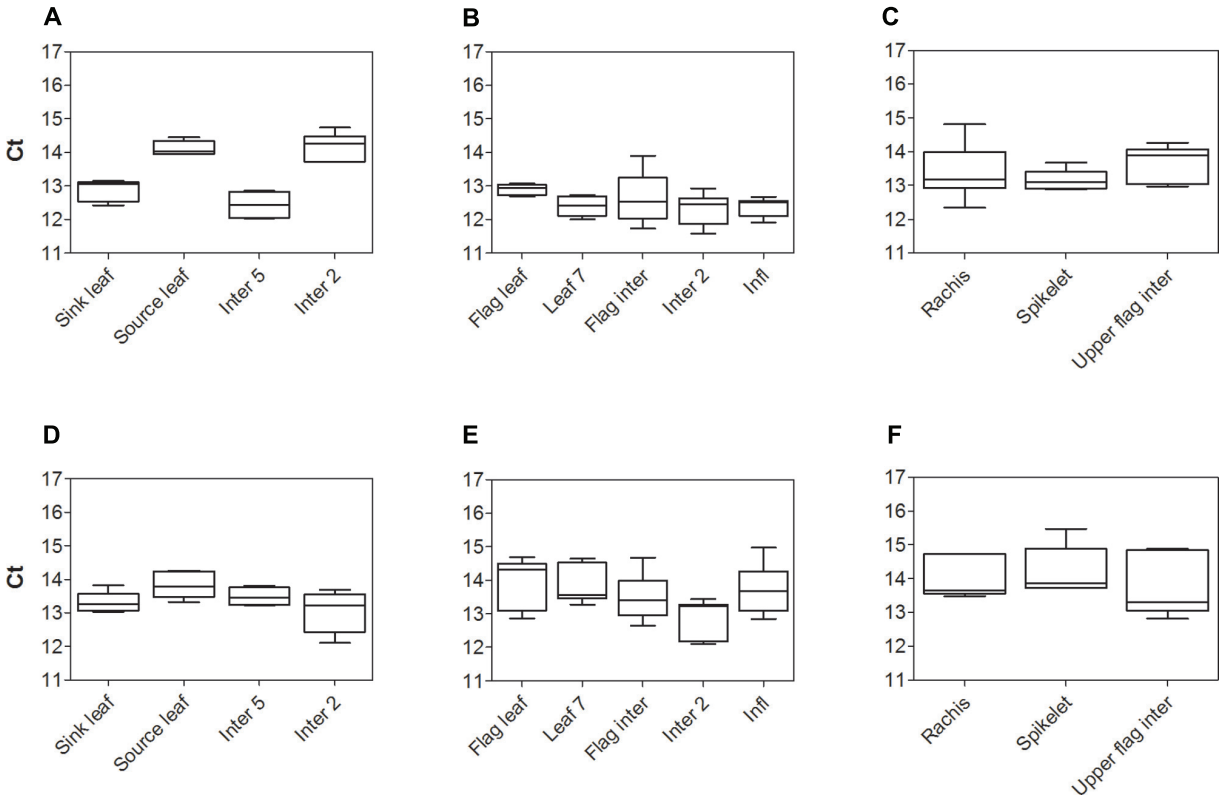

FIGURE 1 | Analysis of *Sorghum* housekeeping gene *SbEF-1α* expression, by qPCR. Cycle threshold (Ct) values for the *Sorghum SbEF-1α* gene in cv. Rio **(A–C)** and cv. BTx623 **(D–F)** during vegetative growth **(A,D)**, at anthesis **(B, E)**, and within the upper flag internode and inflorescence components at anthesis **(C, F)**. Box and whisker plots represent minimum to maximum Ct value, with upper and lower quartile from five biological replicates.

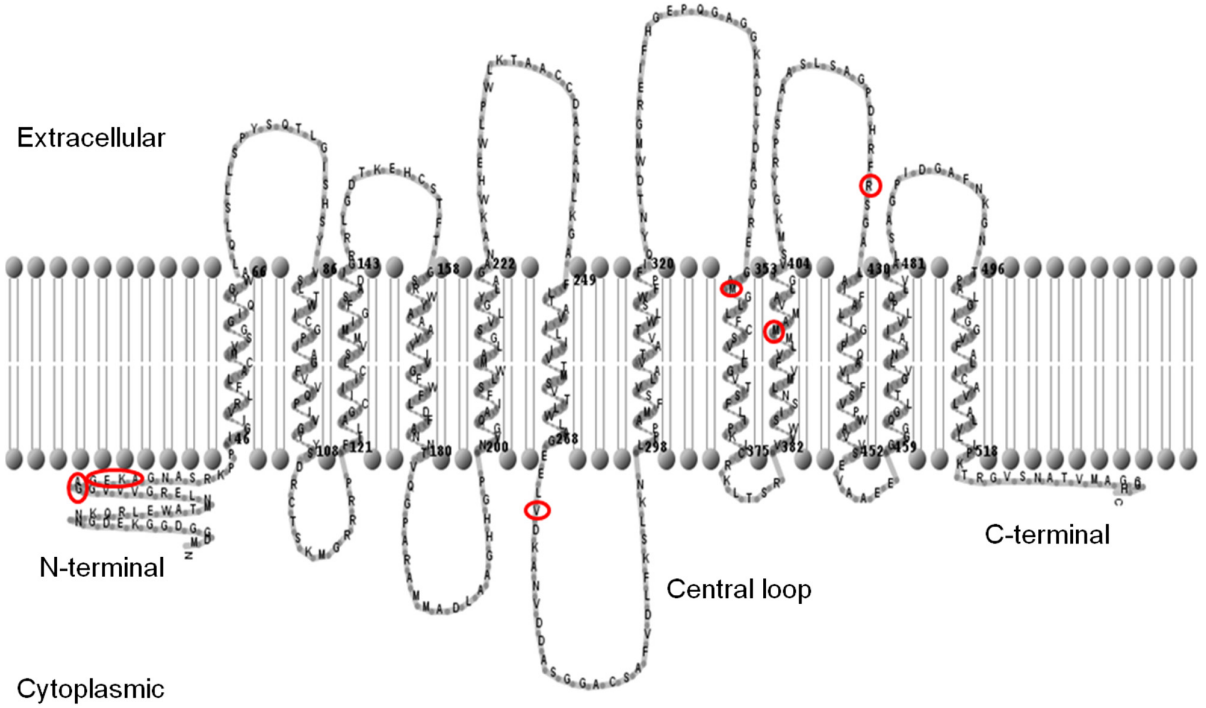

FIGURE 2 | Predicted membrane topology of the SbSUT5 protein. The predicted trans-membrane regions of the SbSUT5 transporter from sweet *Sorghum* (Rio), identifying which amino acids differ between cv. Rio and cv. BTx623 (red circles). Twelve trans-membrane domains are predicted. The membrane topology was generated using the TMHMM server v 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TMRPpres2D viewer (Spyropoulos et al., 2004).

His-65 (Lu and Bush, 1998) and amino acids which correspond to the G-X-X-X-D/E-R/K-X-G-[X]-R/K-R/K motif reside in the second and eighth loop domains (Lemoine, 2000; Pazdernik et al., 2000). Only SbSUT4 contained an LXXLL motif in the N-terminal domain, indicating it may be targeted to the tonoplast (Yamada et al., 2010).

A number of amino acid differences were noted between cv. Rio *SUTs* and the published cv. BTx623 genomic sequence. To examine this further, *SUTs* from cv. BTx623 were cloned and sequences verified. SbSUT1 and SbSUT2 possessed single amino acid sequence differences, whereas SbSUT3, SbSUT4, and SbSUT6 were identical when sequences from cv. Rio and cv. BTx623 were aligned. SbSUT1 from cv. Rio had a valine (V) at position 381, whereas cv. BTx623 had an isoleucine (I) in this position. In SbSUT2 at amino acid 41, a threonine (T) was present in the sequence from cv. Rio, but absent in the cv. BTx623 sequence. SbSUT5 exhibited the most variation between the two cultivars with nine amino acid differences. Five amino acids out of a string of six differed between cv. BTx623 and cv. Rio SUT5, and were predicted to lie in the N-terminal region of the transporter (**Figure 2**). Starting at amino acid 32, the cv. Rio sequence predicted GAGEKA whilst the cv. BTx623 sequence predicted AGEKKG. Single amino acid differences between the cv. Rio and cv. BTx623 sequences occurred at amino acid 272, 355, 396, and 426 as shown in **Figure 2** (V272L; M355V; M396T, and R426K, respectively). These amino acid sequence differences in the SUTs between the two cultivars are summarized in **Table 2**.

Table 2 | Summary of SUT sequence variation between BTx623 and Rio cultivars.

SUT	No. of variations (BTx623 vs Rio)	Amino acid variations
SbSUT1	1	I381V
SbSUT2	1	T41 insertion (Rio)
SbSUT3	0	–
SbSUT4	0	–
SbSUT5	9	A32G, G33A, E34G, K35E, G37A, L272V, V355M, T396M, K426R
SbSUT6	0	–

PHYLOGENETIC ANALYSIS OF MONOCOTYLEDONOUS SUTs

A phylogenetic analysis demonstrated that the *Sorghum* SUTs clustered into four clear groups (**Figure 3**). This is consistent with phylogenetic analyses of other grass species including the C_3, *Lolium perenne* (Berthier et al., 2009) and the C_4 *Zea mays* (Braun and Slewinski, 2009). Two transporters appeared in Groups 1 and 5. In previous studies, Group 2 contained only SUTs from eudicots (Berthier et al., 2009; Braun and Slewinski, 2009). The *Sorghum* SUTs aligned closely with SUTs from other C_4 monocotyledonous species such as maize, sugarcane, and *Setaria viridis* (**Figure 3**).

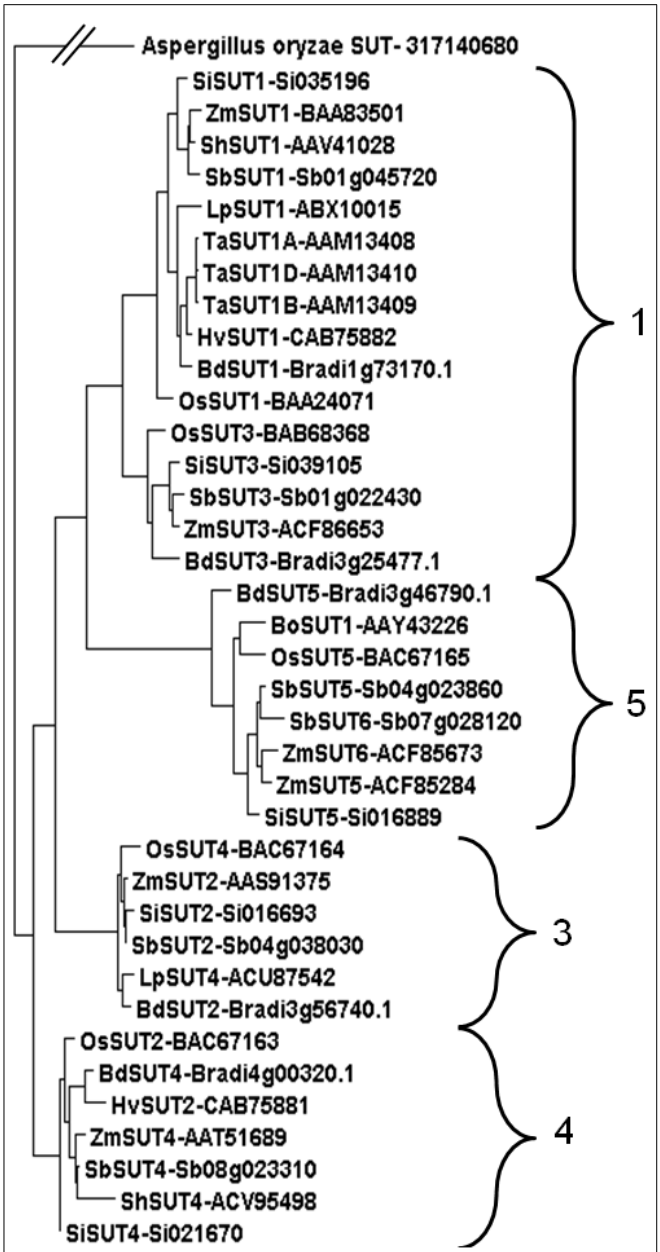

FIGURE 3 | Phylogenetic analysis of SUTs from monocotyledonous species. SUTs displayed fit into Groups 1, 3, 4, 5 (Braun and Slewinski, 2009) from species *Brachypodium distachyon** (BdSUT1, BdSUT2, BdSUT3, BdSUT4, BdSUT5), *Bambusa oldhamii* (BoSUT1), *Hordeum vulgare* (HvSUT1, HvSUT2), *Lolium perenne* (LpSUT1, LpSUT4), *Oryza sativa** (OsSUT1, OsSUT2, OsSUT3, OsSUT4, OsSUT5), *Saccharum* hybrid (ShSUT1, ShSUT4), *Setaria italica** (SiSUT1, SiSUT2, SiSUT3, SiSUT4, SiSUT5), *Sorghum bicolor** (SbSUT1 – Sb01g045720, SbSUT2 – Sb04g038030, SbSUT3 – Sb01g022430, SbSUT4 – Sb08g023310, SbSUT5 – Sb04g023860, SbSUT6 – Sb07g028120), *Triticum aestivum* (TaSUT1A, TaSUT1B, TaSUT1D), *Zea mays** (ZmSUT1, ZmSUT2, ZmSUT3, ZmSUT4, ZmSUT5, ZmSUT6). Phylogenetic analysis was carried out using MUSCLE alignment, Gblocks curation followed by PhyML phylogeny (Dereeper et al., 2008) before viewing in Dendroscope (Huson et al., 2007). Accession numbers are shown along with gene identifications (*Brachypodium*, *Setaria*, and *Sorghum*). Asterisks indicate that the full genomic sequence is publicly available.

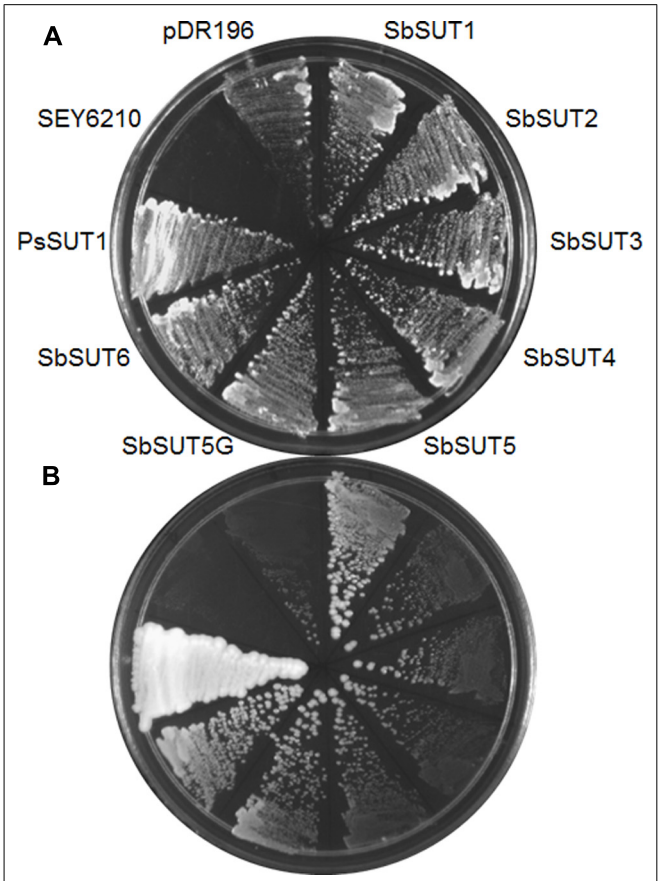

FIGURE 4 | Complementation of the SEY6210 yeast strain by *Sorghum* SUTs. All *Sorghum* SUTs were expressed in the yeast strain SEY6210 and grown on media containing **(A)** 100 mM glucose or **(B)** 25 mM sucrose as the sole carbon source. SuSy7 containing PsSUT1 was used as a positive control. The SuSy7 PsSUT1-pDR195 (Zhou et al., 2007) was used as a positive control and negative controls were untransformed SEY6210 and pDR196 empty vector.

EXPRESSION OF *SUTs* IN YEAST

SUTs from cv. Rio were cloned and expressed in yeast using pDR195 or pDR196 yeast expression vectors (Rentsch et al., 1995), along with the *SbSUT5* from cv. BTx623. The SEY6210 strain of *Saccharomyces cerevisiae* supported growth on media containing sucrose as the sole carbon source, when complemented with each *SUT* (**Figure 4**). This indicates that the introduced SUT mediated sucrose import from the media to support yeast growth.

TRANSCRIPT LEVELS OF *SUTs*

All *SUTs* were expressed at measurable levels in all organs examined apart from *SbSUT3*, consistent with previous observations (Qazi et al., 2012). *SbSUT1* transcripts were detected in both source and sink organs with higher levels observed in cv. BTx623 compared to cv. Rio (two to threefold higher; **Figures 5A** and **6A**). During the vegetative stage of development, fully expanded leaves exhibited the highest level of expression, followed by expanding leaves and stems (**Figure 5A**). At anthesis, fully expanded leaves exhibited substantially higher (fourfold) levels of expression than

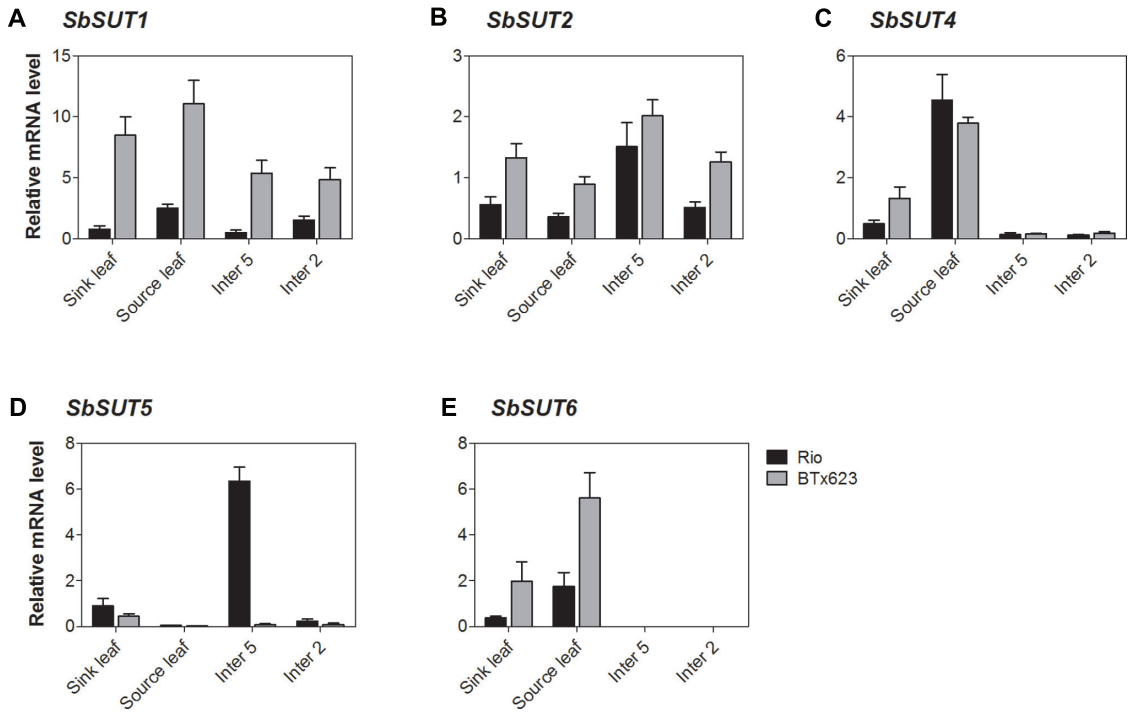

FIGURE 5 | *Sorghum* SUT transcript levels during vegetative growth. Relative expression during vegetative growth of *Sorghum* SUTs. **(A)** *SbSUT1*; **(B)** *SbSUT2*; **(C)** *SbSUT4*; **(D)** *SbSUT5*; **(E)** *SbSUT6*. Levels of *SUT* expression were measured relative to *SbEF-1α*. Organs examined were a Sink leaf (expanding); Source leaf (youngest fully expanded); Internode 5 (Inter 5, elongating); and Internode 2 (Inter 2, fully elongated). Columns with vertical bars represent mean ± SE from five biological replicates.

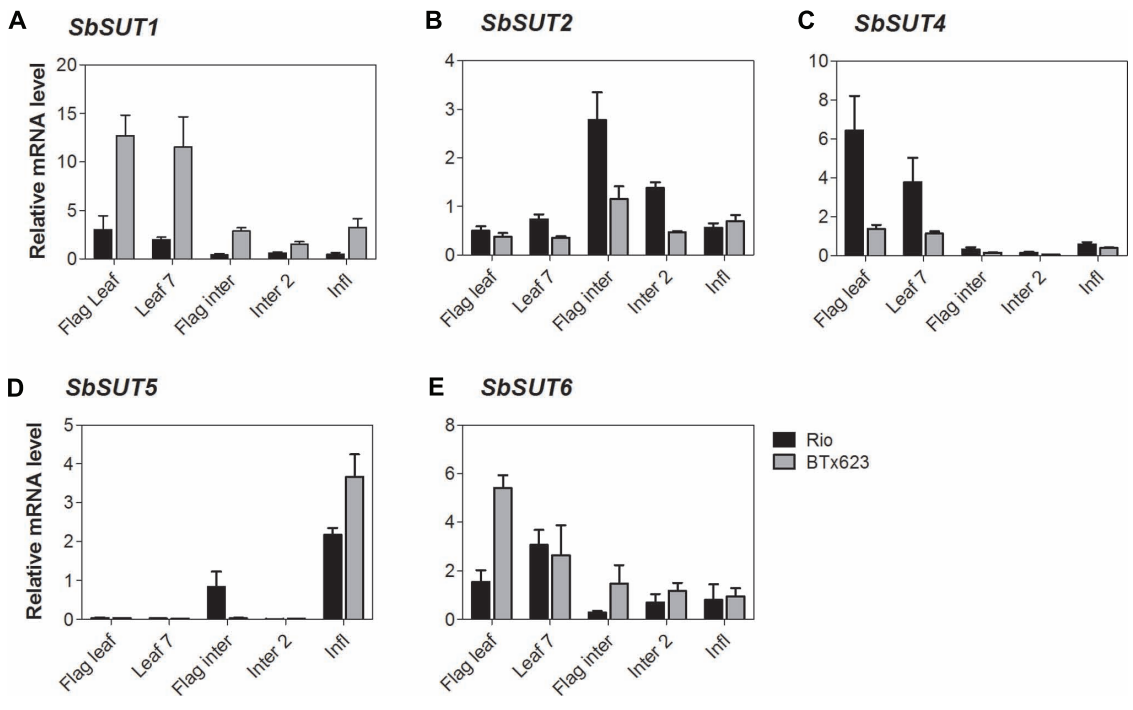

FIGURE 6 | *Sorghum* SUT transcript levels at anthesis. Relative expression at anthesis of *Sorghum* SUTs **(A)** *SbSUT1*; **(B)** *SbSUT2*; **(C)** *SbSUT4*; **(D)** *SbSUT5*; **(E)** *SbSUT6*. Levels of *SUT* expression were measured relative to *SbEF-1α*. Organs examined were the Flag leaf; Leaf 7; flag internode (Flag inter); Internode 2 (Inter 2) and the inflorescence (Infl). Columns with vertical bars represent mean ± SE from five biological replicates.

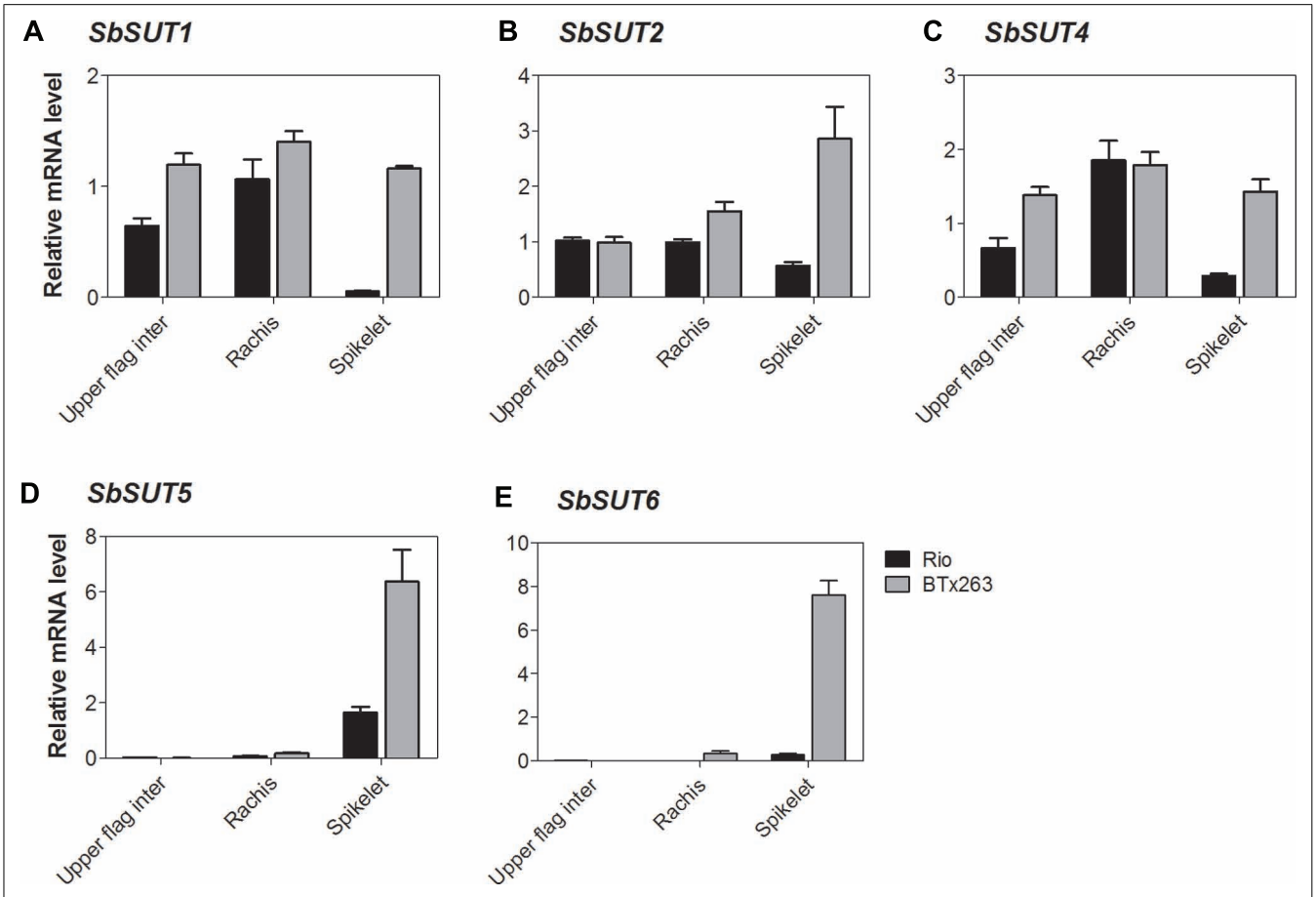

FIGURE 7 | *Sorghum* SUT transcript levels at anthesis in the upper portion of the flag internode and within the inflorescence. Relative expression at anthesis of the *Sorghum* SUTs **(A)** *SbSUT1*; **(B)** *SbSUT2*; **(C)** *SbSUT4*; **(D)** *SbSUT5*; **(E)** *SbSUT6*. Levels of *SUT* expression were measured relative to *SbEF-1α*. Organs examined were the Rachis and Spikelet as well as the upper portion of the flag internode (Upper flag inter). Columns with vertical bars represent mean ± SE from five biological replicates.

stems and inflorescences (**Figure 6A**). Expression levels were similar between cultivars in upper portions of their flag internodes along with rachis branches, but were greater in cv. BTx623 than cv. Rio in spikelets (**Figure 7A**).*SbSUT2* was expressed in all organs examined in both cultivars. During vegetative growth, expression was slightly higher in young elongating stems compared to other organs (**Figure 5B**). At anthesis, flag internodes had the highest levels of *SbSUT2* transcript with cv. Rio being twofold higher than cv. BTx623 (**Figure 6B**). Transcript levels were highest in the cv. BTx623 spikelets exhibiting a threefold difference compared to cv. Rio. Twofold higher levels of expression were observed in spikelets of cv. BTx623 than in rachis branches and upper portions of flag internodes of either cultivar (**Figure 7B**).

During vegetative growth, *SbSUT4* exhibited a similar pattern of expression in the two cultivars. *SbSUT4* expression was highest in fully expanded leaves, with at least twofold lower levels in other organs examined and especially so for stems (**Figure 5C**). In contrast, at anthesis, transcript levels of *SbSUT4* in source leaves were two to threefold greater in cv. Rio compared to cv. BTx623. Within inflorescences, *SbSUT4* transcripts were equally high in rachis branches but for spikelets, expression levels in cv. BTx623 exceeded those of cv. Rio by fourfold (**Figure 7C**). The cultivar difference was reflected, but to a lesser extent, in upper portions of their flag internodes (**Figure 7C**).

There was a clear trend in *SbSUT5* expression during the vegetative stage of development and at anthesis, and expression levels differed between cv. Rio and cv. BTx623 at both developmental stages. During vegetative growth, *SbSUT5* was strongly and exclusively expressed in elongating Internode 5 of cv. Rio (**Figure 5D**). At anthesis, the dominant level of expression switched to inflorescences with cv. BTx623 expression levels exceeding those of cv. Rio by ca 50% (**Figure 6D**). Within inflorescences, *SbSUT5* was expressed primarily in spikelets with threefold higher levels in cv. BTx623 compared to cv. Rio (**Figure 7D**). Transcripts were present in the flag internode of cv. Rio and absent in the same organ of cv. BTx623 (**Figure 6D**).

Transcripts of *SbSUT6* were only detected in sink and source leaves during vegetative growth with levels in cv. BTx623 being threefold greater than those of cv. Rio (**Figure 5E**). At anthesis, leaf expression dominance was retained with cultivar differences declining with leaf age (compare flag and leaf 7 – **Figure 6E**). However, low transcript levels were detected in stems

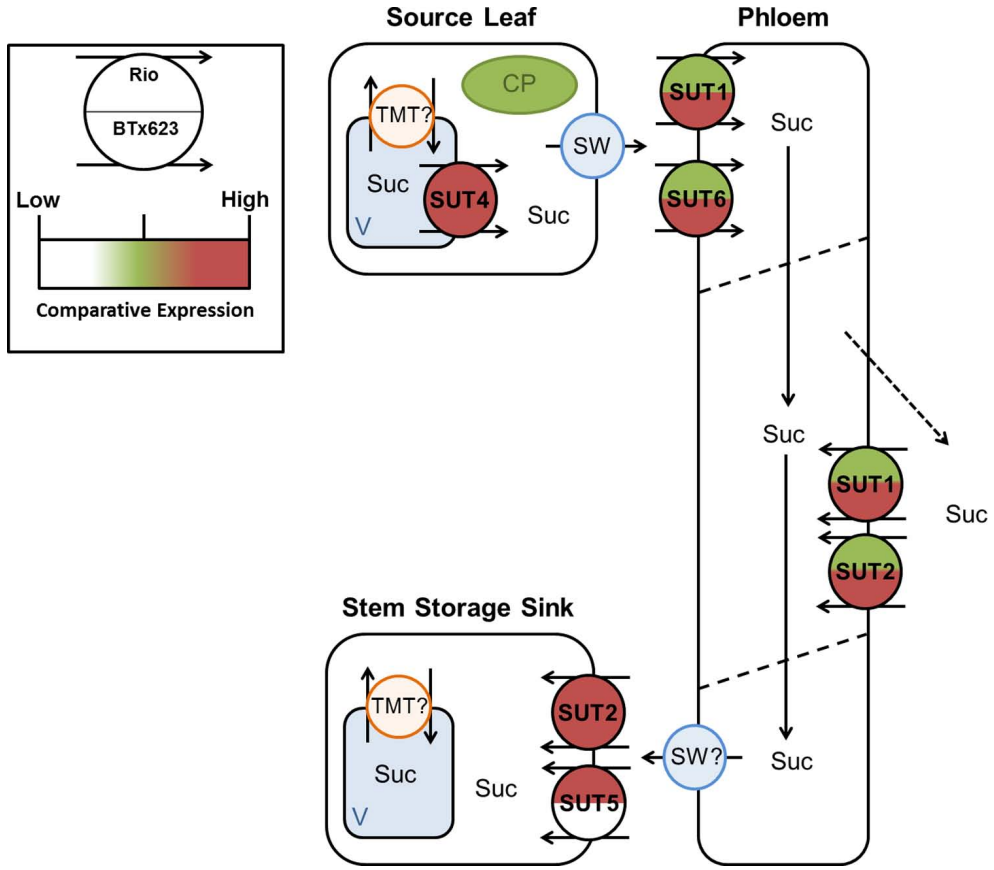

FIGURE 8 | Predicted source–transport–sink pathway in *Sorghum* during vegetative growth. Sucrose is released from source vacuoles (V) by SbSUT4. SbSUT1 and SbSUT6 load the phloem. SbSUT1 and SbSUT2 may act to retrieve sucrose leaked from the transport phloem. SbSUT2 and SbSUT5 load sucrose into stem sinks. SWEETs (SW) efflux sucrose to the apoplasm and tonoplast monosaccharide transporters (TMT) move sucrose into vacuoles. Comparison of relative expression of each SUT is color coded.

and inflorescences (**Figure 6E**). Within inflorescences, *SbSUT6* was strongly expressed in cv. BTx623 spikelets and either weakly expressed or absent from rachis branches and upper portions of flag internodes (**Figure 7E**).

DISCUSSION

The full genomic sequence of *Sorghum* has allowed identification of all *SbSUT* sequences in this model cereal monocot. Examination of *SbSUT* transcript levels in source leaves versus stem and inflorescence sinks provides a strong indication of the role each transporter may play in transporting sucrose from source leaves to these sinks. To further highlight these roles, two phenotypically different cultivars were used, BTx623 and Rio. BTx623 preferentially partitions sucrose to developing inflorescences and hence an emphasis on grain yield whilst cv. Rio stores sucrose in stem parenchyma cells similar to sugarcane. Collectively these analyzes begin to identify which SUTs may participate in phloem loading, axial phloem transport, and phloem unloading.

All six SbSUTs demonstrated complementation of the deficient yeast strain, SEY6210 (**Figure 4**), indicating they are sucrose transport competent, and likely to be functional *in planta*. The single amino acid sequence differences in SbSUT2 sequence between cultivars is predicted to lie in its N-terminal domain, as does the string of amino acids which vary in the SbSUT5 (see **Figure 2** and **Table 2**). The N-terminal domain has been shown to alter SUT affinity for sucrose (Schulze et al., 2000). In addition, recent evidence has identified particular amino acids in rice SUT1 which alters its transport activity (Reinders et al., 2012; Sun et al., 2012). However, these do not appear to correspond with amino acid differences we have identified between cv. BTx623 and cv. Rio (**Figure 2** and **Table 2**). Possible impacts of the detected SbSUT sequence differences between cultivars observed here need to be assessed experimentally.

In terms of phloem loading, based on their relative expression levels in source leaves, identified SbSUT4, SbSUT1, and SbSUT6 as potential candidates during vegetative and reproductive growth (see **Figures 5, 6, 8,** and **9**). For SbSUT4, this assertion is consistent with a high source leaf expression observed for *OsSUT2* (Eom et al., 2011) and *Populus tremula* × *alba* (gray poplar) *PtaSUT4* (Payyavula et al., 2011). A number of transporters belonging to the same phylogenetic group as SbSUT4 (see **Figure 3**), have been localized to the tonoplast. These include rice SUT2 (Eom et al., 2011), barley SUT2 (Endler et al., 2006; Group 4) and the dicotyledonous SUTs, AtSUT4 (Endler et al.,

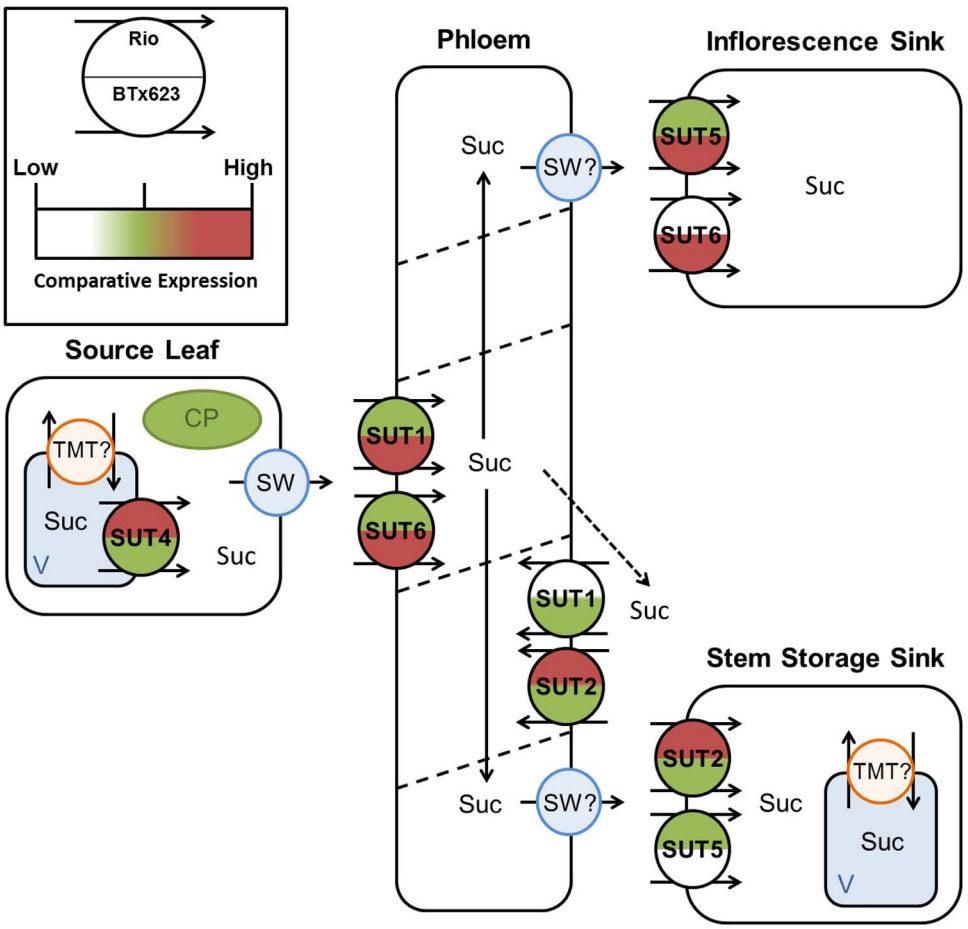

FIGURE 9 | Predicted source–transport–sink pathway in *Sorghum* at anthesis. Sucrose is released from source vacuoles (V) by SbSUT4. SbSUT1 and SbSUT6 load the phloem. SbSUT1 and SbSUT2 may act to retrieve sucrose leaked from the transport phloem. SbSUT2 and SbSUT5 load sucrose into stem sinks. SbSUT5 and SbSUT6 load sucrose into reproductive sinks. SWEETs (SW) efflux sucrose to the apoplasm and tonoplast monosaccharide transporters (TMT) move sucrose into vacuoles. Comparison of relative expression of each SUT is color coded.

2006), *Lotus japonicus* LjSUT4 (Reinders et al., 2008), poplar PtaSUT4 (Payyavula et al., 2011), and tobacco NtSUT4 (Okubo-Kurihara et al., 2011). On the tonoplast, SUT4 functions to release sucrose from mesophyll vacuoles to their cytoplasm (Schulz et al., 2011; Schneider et al., 2012) rendering the vacuolar pool of sucrose available for phloem loading. The significance of this function for SUT4 is demonstrated by slowed photoassimilate export in knock down SUT4 mutants of rice (Eom et al., 2011).

SbSUT1 may play a role in apoplasmic phloem loading (**Figures 8** and **9**) as found for the closely related maize ZmSUT1 which also belongs to the C_4 NADP-ME subgroup (Slewinski et al., 2009). This assertion is based on finding that a maize *sut1* mutant exhibited a phenotype of shorter stature and carbohydrate accumulation in their source leaves (Slewinski et al., 2009). Consistent with this phenotype, the *sut1* mutant had a diminished ability to export sucrose from source leaves. Greater levels of *SbSUT1* transcript were detected in source leaves of cv. BTx623 than cv. Rio (**Figures 5A** and **6A**). These differences could reflect differences in sink demand between cultivars driving photosynthetic rate along with sucrose export from source leaves (Minchin et al., 2002; McCormick et al., 2006). *SbSUT6* is another phloem loading candidate (**Figures 8** and **9**). Similar to *SbSUT1*, transcript levels of *SbSUT6* were higher in source leaves of cv. BTx623 than cv. Rio at both vegetative and anthesis stages (**Figures 5E** and **6E**) supporting the notion that sink demand might be stronger in cv. BTx623.

In sweet *Sorghum*, sucrose is radially transferred from the phloem into stem storage parenchyma cells through a post-sieve element unloading pathway that likely includes an apoplasmic step (Tarpley and Vietor, 2007). In this context, *SbSUT2* and *SbSUT5* were highly expressed in internodes (**Figures 5B** and **6B**) where they may play a primary role in phloem unloading, retrieval of sucrose leaked from the phloem or phloem loading of sucrose remobilized from stem storage (**Figures 8** and **9**). However, since remobilization predominantly occurs during grain filling (Gutjahr et al., 2013), at anthesis SUTs likely function to facilitate radial transport of sucrose to stem storage pools or in retrieving sucrose leaked from the phloem.

Twofold greater expression of *SbSUT2* was observed in internodes of cv. Rio as opposed to those from cv. BTx623 at anthesis

(**Figure 6D**), suggesting this SUT may play an enhanced role in directing sucrose to stem storage parenchyma cells in cv. Rio. SbSUT2 has a predicted protein sequence of 594 amino acids, which is at least 60 amino acids longer than the five other *Sorghum* SUTs. Similarly, OsSUT4 has an extended central loop domain of around 90 amino acids and an extended N-terminal domain (Aoki et al., 2003). Little information is available for the other monocot Group 3 SUTs. However, an insertional mutant of the corresponding dicot SUT AtSUT3, showed no morphological phenotype (Barth et al., 2003).In comparison to the strong expression of *SbSUT5* in cv. Rio internodes (**Figures 5D** and **6D**), *SbSUT5* transcripts were absent from cv. BTx623 internodes. Rather *SbSUT5* transcripts were 1.5–2-fold higher in inflorescences of cv. BTx623 than cv. Rio (**Figure 6D**), and especially spikelets (**Figure 7D**). These expression patterns suggest that cv. BTx623 directs more sucrose toward development of reproductive structures than cv. Rio. *SbSUT6*, most closely related to *SbSUT5* (**Figure 3**), was strongly expressed within cv. BTx623 spikelets (**Figure 7E**) and hence may also contribute to inflorescence development (**Figure 9**).

Cultivar differences in expression profiles of *SbSUT5* were accompanied by the highest number of amino acid difference in SbSUT5 sequences (9 amino acids, **Table 2**). Little information is available about transporters belonging to Group 5 apart from *OsSUT5*. This gene exhibited broad expression across source and sink leaves as well as in developing grains of rice (Aoki et al., 2003). Similar to *SbSUT5*, SUT1 transporters of more distantly related C_3 species of rice, barley, and wheat appear to play a role in grain development. Antisense lines for *OsSUT1* showed little phenotypic difference when compared to wild-type in vegetative growth or differences in carbohydrate content of their source leaves (Ishimaru et al., 2001). However, grain filling was reduced substantially in *OsSUT1* antisense plants (Scofield et al., 2002). A number of other monocotyledonous plant SUT1 transporters also were found to be involved in grain development, including barley (Weschke et al., 2000) and wheat (Aoki et al., 2002) SUT1-type proteins. Whether *SbSUT1* plays a major role in grain development remains to be determined.

In conclusion, the six *Sorghum SUTs* were cloned from two cultivars that differ in carbohydrate partitioning. Expression analysis revealed that three of the *SUTs* were expressed strongly in source leaves (*SbSUT1*, *SbSUT4*, *SbSUT6*) and are likely to play roles in phloem loading. Two *SUTs* were expressed strongly in sinks (*SbSUT2*, *SbSUT5*) and are more likely to play roles in sink development and photoassimilate storage. *SbSUT3* was not detected in most organs examined. All of the *Sorghum* SUTs complemented the deficient yeast system, indicating they are sucrose transport competent. A number of amino acid sequence variations were identified between the SUTs from the two cultivars, and future functional characterization will determine if these variations result in alteration of their sucrose transport properties.

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REFERENCES

Aoki, N., Hirose, T., Scofield, G. N., Whitfeld, P. R., and Furbank, R. T. (2003). The sucrose transporter gene family in rice. *Plant Cell Physiol.* 44, 223–232. doi: 10.1093/pcp/pcg030

Aoki, N., Whitfield, P., Hoeren, F., Scofield, G., Newell, K., Patrick, J., et al. (2002). Three sucrose transporter genes are expressed in the developing grain of hexaploid wheat. *Plant Mol. Biol.* 50, 453–462. doi: 10.1023/A:1019846832163

Barth, I., Meyer, S., and Sauer, N. (2003). PmSUC3: characterization of a SUT2/SUC3-type sucrose transporter from *Plantago major*. *Plant Cell* 15, 1375–1385. doi: 10.1105/tpc.010967

Berthier, A., Desclos, M., Amiard, V., Morvan-Bertrand, A., Demmig-Adams, B., Adams, W. W., et al. (2009). Activation of sucrose transport in defoliated *Lolium perenne* L.: an example of apoplastic phloem loading plasticity. *Plant Cell Physiol.* 50, 1329–1344. doi: 10.1093/pcp/pcp081

Blum, A., Sinmena, B., Mayer, J., Golan, G., and Shpiler, L. (1994). Stem reserve mobilization supports wheat-grain filling under heat-stress. *Aust. J. Plant Physiol.* 21, 771–781. doi: 10.1071/PP9940771

Blum, A., Golan, G., Mayer, J., and Sinmena, B. (1997). The effect of dwarfing genes on *sorghum* grain filling from remobilized stem reserves, under stress. *Field Crops Res.* 52, 43–54. doi: 10.1016/S0378-4290(96)03462-4

Braun, D. M., and Slewinski, T. L. (2009). Genetic control of carbon partitioning in grasses: roles of sucrose transporters and tie-dyed loci in phloem loading. *Plant Physiol.* 149, 71–81. doi: 10.1104/pp.108.129049

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469. doi: 10.1093/nar/gkn180

Dohmen, R. J., Strasser, A. W. M., Honer, C. B., and Hollenberg, C. P. (1991). An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. *Yeast* 7, 691–692. doi: 10.1002/yea.320070704

Endler, A., Meyer, S., Schelbert, S., Schneider, T., Weschke, W., Peters, S. W., et al. (2006). Identification of a vacuolar sucrose transporter in barley and *Arabidopsis* mesophyll cells by a tonoplast proteomic approach. *Plant Physiol.* 141, 196–207. doi: 10.1104/pp.106.079533

Eom, J. S., Cho, J. I., Reinders, A., Lee, S. W., Yoo, Y., Tuan, P. Q., et al. (2011). Impaired function of the tonoplast-localized sucrose transporter in rice, OsSUT2, limits the transport of vacuolar reserve sucrose and affects plant growth. *Plant Physiol.* 157, 109–119. doi: 10.1104/pp.111.176982

Gutjahr, S., Vaksmann, M., Dingkuhn, M., Thera, K., Trouche, G., Braconnier, S., et al. (2013). Grain, sugar and biomass accumulation in tropical sorghums. I. Trade-offs and effects of phenological plasticity. *Funct. Plant Biol.* 40, 342–354. doi: 10.1071/FP12269

Hoffmann-Thoma, G., Hinkel, K., Nicolay, P., and Willenbrink, J. (1996). Sucrose accumulation in sweet *sorghum* stem internodes in relation to growth. *Physiol. Plant.* 97, 277–284. doi: 10.1034/j.1399-3054.1996.970210.x

Huson, D. H., Richter, D. C., Rausch, C., Dezulian, T., Franz, M., and Rupp, R. (2007). Dendroscope: an interactive viewer for large phylogenetic trees. *BMC Bioinformatics* 8:460. doi: 10.1186/1471-2105-8-460

Ishimaru, K., Hirose, T., Aoki, N., Takahashi, S., Ono, K., Yamamoto, S., et al. (2001). Antisense expression of a rice sucrose transporter OsSUT1 in rice (*Oryza sativa* L.). *Plant Cell Physiol.* 42, 1181–1185. doi: 10.1093/pcp/pce148

Jacobsen, K. R., Fisher, D. G., Maretzki, A., and Moore, P. H. (1992). Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Bot. Acta* 105, 70–80.

Lalonde, S., Wipf, D., and Frommer, W. B. (2004). Transport mechanisms for organic forms of carbon and nitrogen between source and sink. *Annu. Rev. Plant Biol.* 55, 341–372. doi: 10.1146/annurev.arplant.55.031903.141758

Lemoine, R. (2000). Sucrose transporters in plants: update on function and structure. *Biochim. Biophys. Acta* 1465, 246–262. doi: 10.1016/S0005-2736(00)00142-5

Lingle, S. E. (1987). Sucrose metabolism in the primary culm of sweet sorghum during development. *Crop Sci.* 27, 1214–1219. doi: 10.2135/cropsci1987.0011183X002700060025x

Lu, J. M. Y., and Bush, D. R. (1998). His-65 in the proton-sucrose symporter is an essential amino

acid whose modification with site-directed mutagenesis increases transport activity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9025–9030. doi: 10.1073/pnas.95.15.9025

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2006). Sink strength regulates photosynthesis in sugarcane. *New Phytol.* 171, 759–770. doi: 10.1111/j.1469-8137.2006.01785.x

Minchin, P. E. H., Thorpe, M. R., Farrar, J. F., and Koroleva, O. A. (2002). Source–sink coupling in young barley plants and control of phloem loading. *J. Exp. Bot.* 53, 1671–1676. doi: 10.1093/jxb/erf003

Murray, S. C., Rooney, W. L., Mitchell, S. E., Sharma, A., Klein, P. E., Mullet, J. E., et al. (2008). Genetic improvement of *Sorghum* as a biofuel feedstock: II. QTL for stem and leaf structural carbohydrates. *Crop Sci.* 48, 2180–2193. doi: 10.2135/cropsci2008.01.0068

Okubo-Kurihara, E., Higaki, T., Kurihara, Y., Kutsuna, N., Yamaguchi, J., and Hasezawa, S. (2011). Sucrose transporter NtSUT4 from tobacco BY-2 involved in plant cell shape during miniprotoplast culture. *J. Plant Res.* 124, 395–403. doi: 10.1007/s10265-010-0377-7

Payyavula, R. S., Tay, K. H. C., Tsai, C. J., and Harding, S. A. (2011). The sucrose transporter family in *Populus*: the importance of a tonoplast PtaSUT4 to biomass and carbon partitioning. *Plant J.* 65, 757–770. doi: 10.1111/j.1365-313X.2010.04463.x

Pazdernik, N. J., Matzke, E. A., Jessen-Marshall, A. E., and Brooker, R. J. (2000). Roles of charged residues in the conserved motif, G-X-X-X-D/E-R/K-X-G-[X]-R/K-R/K, of the lactose permease of *Escherichia coli*. *J. Membr. Biol.* 174, 31–40. doi: 10.1007/s002320001029

Preisser, J., and Komor, E. (1991). Sucrose uptake into vacuoles of sugarcane suspension cells. *Planta* 186, 109–114. doi: 10.1007/BF00201505

Qazi, H. A., Paranjpe, S., and Bhargava, S. (2012). Stem sugar accumulation in sweet *sorghum* – activity and expression of sucrose metabolizing enzymes and sucrose transporters. *J. Plant Physiol.* 169, 605–613. doi: 10.1016/j.jplph.2012.01.005

Reinders, A., Sivitz, A. B., Starker, C. G., Gantt, J. S., and Ward, J. M. (2008). Functional analysis of LjSUT4, a vacuolar sucrose transporter from *Lotus japonicus*. *Plant Mol. Biol.* 68, 289–299. doi: 10.1007/s11103-008-9370-0

Reinders, A., Sun, Y., Karvonen, K. L., and Ward, J. M. (2012). Identification of amino acids important for substrate specificity in sucrose transporters using gene shuffling. *J. Biol. Chem.* 287, 30296–30304. doi: 10.1074/jbc.M112.372888

Rentsch, D., Laloi, M., Rouhara, I., Schmelzer, E., Delrot, S., and Frommer, W. B. (1995). NTR1 encodes a high-affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett.* 370, 264–268. doi: 10.1016/0014-5793(95)00853-2

Robinson, J. S., Klionsky, D. J., Banta, L. M., and Emr, S. D. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8, 4936–4948.

Saftner, R. A., Daie, J., and Wyse, R. E. (1983). Sucrose uptake and compartmentation in sugar-beet taproot tissue. *Plant Physiol.* 72, 1–6. doi: 10.1104/pp.72.1.1

Schneider, S., Hulpke, S., Schulz, A., Yaron, I., Holl, J., Imlau, A., et al. (2012). Vacuoles release sucrose via tonoplast-localised SUC4-type transporters. *Plant Biol.* 14, 325–336. doi: 10.1111/j.1438-8677.2011.00506.x

Schulz, A., Beyhl, D., Marten, I., Wormit, A., Neuhaus, E., Poschet, G., et al. (2011). Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2. *Plant J.* 68, 129–136. doi: 10.1111/j.1365-313X.2011.04672.x

Schulze, W., Weise, A., Frommer, W. B., and Ward, J. M. (2000). Function of the cytosolic N-terminus of sucrose transporter AtSUT2 in substrate affinity. *FEBS Lett.* 485, 189–194. doi: 10.1016/S0014-5793(00)02180-3

Scofield, G. N., Hirose, T., Gaudron, J. A., Upadhyaya, N. M., Ohsugi, R., and Furbank, R. T. (2002). Antisense suppression of the rice sucrose transporter gene, OsSUT1, leads to impaired grain filling and germination but does not affect photosynthesis. *Funct. Plant Biol.* 29, 815–826. doi: 10.1071/PP01204

Slewinski, T. L., Meeley, R., and Braun, D. M. (2009). Sucrose transporter1 functions in phloem loading in maize leaves. *J. Exp. Bot.* 60, 881–892. doi: 10.1093/jxb/ern335

Spyropoulos, I. C., Liakopoulos, T. D., Bagos, P. G., and Hamodrakas, S. J. (2004). TMRPres2D: high quality visual representation of trans-membrane protein models. *Bioinformatics* 20, 3258–3260. doi: 10.1093/bioinformatics/bth358

Sun, Y., Lin, Z., Reinders, A., and Ward, J. M. (2012). Functionally important amino acids in rice sucrose transporter OsSUT1. *Biochemistry* 51, 3284–3291. doi: 10.1021/bi201934h

Tarpley, L., and Vietor, D. M. (2007). Compartmentation of sucrose during radial transfer in mature *sorghum* culm. *BMC Plant Biol.* 7:33. doi: 10.1186/1471-2229-7-33

Weschke, W., Panitz, R., Sauer, N., Wang, Q., Neubohn, B., Weber, H., et al. (2000). Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *Plant J.* 21, 455–467. doi: 10.1046/j.1365-313x.2000.00695.x

Williams, L., Thom, M., and Maretzki, A. (1990). Characterization of a proton translocating ATPase and sucrose uptake in a tonoplast-enriched vesicle fraction from sugarcane. *Physiol. Plant.* 80, 169–176. doi: 10.1111/j.1399-3054.1990.tb04392.x

Yamada, K., Osakabe, Y., Mizoi, J., Nakashima, K., Fujita, Y., Shinozaki, K., et al. (2010). Functional analysis of an *Arabidopsis thaliana* abiotic stress-inducible facilitated diffusion transporter for monosaccharides. *J. Biol. Chem.* 285, 1138–1146. doi: 10.1074/jbc.M109.054288

Zhao, Y. L., Dolat, A., Steinberger, Y., Wang, X., Osman, A., and Xie, G. H. (2009). Biomass yield and changes in chemical composition of sweet *sorghum* cultivars grown for biofuel. *Field Crops Res.* 111, 55–64. doi: 10.1016/j.fcr.2008.10.006

Zhou, Y. C., Qu, H. X., Dibley, K. E., Offler, C. E., and Patrick, J. W. (2007). A suite of sucrose transporters expressed in coats of developing legume seeds includes novel pH-independent facilitators. *Plant J.* 49, 750–764. doi: 10.1111/j.1365-313X.2006.03000.x

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Proteomic comparison of basal endosperm in maize *miniature*1 mutant and its wild-type *Mn*1

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Developing endosperm in maize seed is a major site for biosynthesis and storage of starch and proteins, and of immense economic importance for its role in food, feed and biofuel production. The basal part of endosperm performs a major role in solute, water and nutrition acquisition from mother plant to sustain these functions. The *miniature1* (*mn*1) mutation is a loss-of-function mutation of the *Mn*1-encoded cell wall invertase that is entirely expressed in the basal endosperm and is essential for many of the metabolic and signaling functions associated with metabolically released hexose sugars in developing endosperm. Here we report a comparative proteomic study between *Mn*1 and *mn*1 basal endosperm to better understand basis of pleiotropic effects on many diverse traits in the mutant. Specifically, we used iTRAQ based quantitative proteomics combined with Gene Ontology (GO) and bioinformatics to understand functional basis of the proteomic information. A total of 2518 proteins were identified from soluble and cell wall associated protein (CWAP) fractions; of these 131 proteins were observed to be differentially expressed in the two genotypes. The main functional groups of proteins that were significantly different were those involved in the carbohydrate metabolic and catabolic process, and cell homeostasis. The study constitutes the first proteomic analysis of basal endosperm cell layers in relation to endosperm growth and development in maize.

Keywords: proteomics, developing endosperm, iTRAQ, gene ontology, maize

INTRODUCTION

Proteomic analysis aims to measure the expression and modification of all proteins in an organism as a function of a number of variants, including environmental conditions, biotic or abiotic stresses, wild type and mutant genotypes, developmental stages etc. (Chen and Harmon, 2006; Lan et al., 2011; Neilson et al., 2011; Owiti et al., 2011). Advancement in mass spectrometry, genome sequencing and bioinformatics has been major impetuses to high-throughput analyses in proteome studies (Miernyk and Hajduch, 2011). Of many plant materials used for proteomic studies, developing seeds are of particular interest because of their economic significance; additionally, they undergo many distinctive phases where each stage is marked by unique cellular or metabolic activity. Among the most recent seed proteomic studies, Lee and Koh (2011) described rice grain profiles at three developmental stages against fully mature grains to show 52 categories out of a total of 4172 proteins. Koller et al. (2002) compared rice proteomes of leaf, root and seed showing 622 leaf-specific, 862 root-specific and 512 seed-specific proteins. The assignment of function based on BLAST searches showed that 360 proteins have no homology to known proteins; thus, these were labeled as rice-specific proteins. A similar study in maize endosperm (Méchin et al., 2007) at seven developmental stages showed a total of 632 proteins; of these 496 were assigned to functional identification. A relative increase in the levels of glycolytic enzymes as compared to TCA enzymes in this study confirmed the previously demonstrated anoxic conditions of the endosperm. It also pointed to a crucial role for pyruvate orthophosphate dikinase in maintaining starch-protein balance during endosperm development.

Although these data in maize are important, they do not take into account that an endosperm is a heterogeneous tissue comprised of at least four known cell types (Scanlon and Takacs, 2009). Of these four, one cell type, basal endosperm transfer layer (BETL), is unique to the basal 1/3rd part of the endosperm that is adjacent to the maternal pedicel (**Figure 1**), and is primarily engaged in acquisition of water, nutrients and solutes from the mother plant. In the context of basal endosperm, the *miniature*1 (*mn*1) seed mutant is of particular interest because it is a loss-of-function mutation of the *Mn*1 gene that encodes an endosperm-specific cell wall invertase (INCW2), which is entirely BETL-specific in its cellular localization (Cheng et al., 1996). Incoming sucrose to a sink tissue such as developing seed is irreversibly hydrolyzed by INCWs to glucose and fructose that is critical for numerous metabolic and signaling functions. In maize endosperm, the INCW2 is spatially and temporally the first enzyme to metabolize sucrose. Homozygous *mn*1 mutant

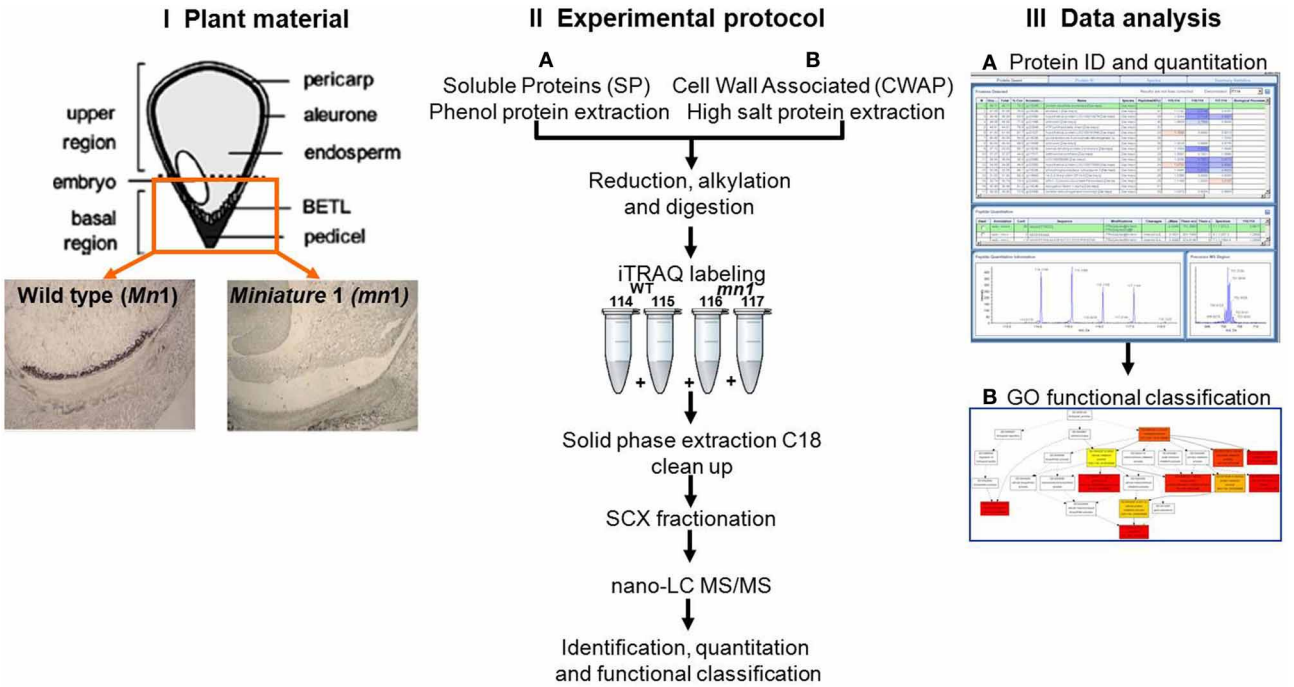

FIGURE 1 | Schematic experimental work flow: (I) Plant materials of *Mn*1 (wild type) and *mn*1 mutant enriched BETL regions; (II) SPs and CWAPs sample preparation, iTRAQ two-dimensional LC-MS/MS; and (III) data analysis.

is non-lethal due to a residual low level (<1% of the total activity) of INCW activity by the co-ortholog, *Incw*1 (Chourey et al., 2006). Not surprisingly, the *mn*1 mutant is associated with changes at both morphological (Miller and Chourey, 1992) and cellular (Vilhar et al., 2002) levels, which include retarded development of wall-in-growths (WIGs)—labyrinth-like growth in plasma membrane area believed to be essential to transport capacity of the BETL and the normal development of seed (Kang et al., 2009). The mutant is also associated with an altered IAA homeostasis (LeClere et al., 2008, 2010) and with greatly altered sugar metabolism, especially in the basal region that shows increased sucrose levels and greatly reduced hexose sugars due to its INCW2-deficiency (LeClere et al., 2010; Chourey et al., 2012). Reductions in both IAA and hexose sugars that are signaling molecules led to changes in the expression of several genes related to sucrose-starch and sucrose-energy metabolism in the *mn*1 mutant (Chourey et al., 2012). Xiong et al. (2011) reported 454 transcriptome sequencing of cDNAs to show 2473 unique sequences from *Mn*1 and *mn*1 BETLs. Functional annotation and categorization analyses of these cDNAs led them to conclude high abundance of transcripts related to mitochondrial activity, alkaloid biosynthesis, and various signaling processes of seed development. Transcriptomic studies however do not often provide a true representation of protein abundance due to the well-known phenomenon of poor concordance between RNA and the corresponding proteins as shown in seed filling in Arabidopsis (Hajduch et al., 2010).

The objectives of this study are 2-fold: (1) to analyze global proteome changes in the basal region of 12 DAP endosperm (i.e., BETL enriched) of the *mn*1 mutant relative to the *Mn*1 to describe or catalog proteins that may be metabolically co-regulated in response to the invertase deficiency. Reduced hexose levels may also activate many genes associated with untranslated protein response (UPR) stress. (2) In addition to soluble proteins (SPs), we also examined ionically-bound proteins presumably enriched for cell wall associate proteins (CWAPs). Because of the many cellular level changes in the *mn*1 basal endosperm (Kang et al., 2009) (most notably the changes in the WIG, which is predominantly comprised of initial primary cell wall and secondary cell wall formation during BETL maturation), we hypothesize that there will be interesting changes in the profiles of proteins associated with cell wall metabolism.

MATERIALS AND METHODS

PLANT MATERIALS AND CHEMICALS

Immature maize (*Zea mays* L.) kernels of *Mn*1, and *mn*1 (Cheng and Chourey, 1999) in the W22 inbred line were harvested at 12 days after pollination (DAP). All plants were grown in the field and were self- or sib-pollinated. At the time of harvest kernels were individually excised from the ear with a paring knife, taking care to include undamaged base (pedicel) of each kernel. Excised kernels were flash frozen in liquid nitrogen and stored at −80°C until analysis. After removing embryos of each kernel, we used the basal 1/3 end of the endosperm because it is specifically enriched in the BETL cells, the sole site of the *Mn*1 expression and also a major zone for sucrose turn-over reactions, as discussed previously (LeClere et al., 2008). Chemicals were purchased from Fisher Scientific Inc., USA unless otherwise stated.

SOLUBLE PROTEIN EXTRACTION

A total of four biological samples, two each of the *Mn*1 and *mn*1 genotypes, and each sample comprised of 5 g of fresh tissue of basal 1/3 endosperm was dissected-out for SP extractions according to a previous method reported by Hurkman and Tanaka (1986) with minor modifications. Care was taken to save aliquots of the same kernel samples for the RNA analyses described below. Each frozen sample was ground into a fine powder with a pre-chilled mortar and pestle. A total of 3 mL of extraction buffer (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.2 M DTT, 0.9 M sucrose) and 3 mL of saturated phenol were added to homogenize the sample; and to extract proteins for 2 h at room temperature in constant agitation. The mixture was then centrifuged at 5000× g for 10 min at 4°C. The top clear phenol phase was removed and collected in a fresh tube. The remaining pellet was re-extracted with 3 mL buffered phenol solution and centrifuged again to recover the top clear phenol phase and combined with the previous one. The supernatant was precipitated with 5 volumes of ice cold 0.1 M ammonium acetate in 100% methanol overnight and centrifuged at 20000× g for 20 min at 4°C. The supernatant was discarded and the pellet was washed twice with 0.1 M ammonium acetate in 100% methanol, then washed twice with cold 80% acetone and finally with 70% of ethanol. The pellet was solubilized in a buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS and 40 mM DTT. The samples were treated with benzonase (Novagen, Gibbstown, NJ) for 30 min and then ultracentrifuged at 34000 rpm for 30 min at 15°C. Supernatant was collected and 50 μL aliquots were kept at −80°C until use.

CELL WALL ASSOCIATED PROTEIN EXTRACTION

In addition to the SP extraction, a high salt extraction was performed according to Cheng et al. (1996), to obtain cell wall associated proteins (CWAPs). Briefly, frozen kernels were homogenized in extraction buffer (50 mM Tris-maleate, pH 7.0 and 1 mM DTT) in a 1:10 (w/v) ratio by using a cooled mortar and pestle. The homogenate was centrifuged at 14000× g for 10 min. The supernatant was discarded and pellet was washed three times in the extraction buffer followed by a final resuspension in extraction buffer containing 1 M NaCl in a 1:2 (w/v) ratio. The salt suspension was vortexed for 30 min at 4°C and then centrifuged at 14000× g for 10 min. The supernatant was recovered and precipitated in a ratio 1:5 with 100% cold acetone overnight. Protein was recovered by centrifugation at 20000× g, 4°C for 30 min. The pellet was washed three times with cold acetone, followed by solubilization and storage as described for the SPs samples.

iTRAQ LABELING AND LC-MS/MS

The SPs and CWAPs samples were quantified using an EZQ Protein Quantitation kit (Invitrogen; USA) (Zhu et al., 2010). A total of 100 μg of total protein from each sample was acetone precipitated. The samples were dissolved in 1% SDS, 100 mM triethylammonium bicarbonate, pH 8.5; then reduced, alkylated, trypsin (Promega, USA) digested and labeled according to manufacturer's instructions (ABsciex Inc. USA). The replicates of *Mn*1 samples were labeled with 114 and 115, whereas the replicates of *mn*1 samples were labeled with 116 and 117. Extra labels were quenched by adding 100 μL of ultrapure water and left at room temperature for 30 min. After quenching, samples were mixed together and dried down in speedvac. The peptide mixtures were cleaned up with C18 spin columns according to manufacturer's instructions (ABsciex Inc. USA). The samples were then dissolved in strong cation exchange (SCX) solvent A (25% v/v ACN, 10 mM ammonium formate, pH 2.8) and injected to a Agilent HPLC 1100 system using a polysulfoethyl A column (2.1 × 100 mm, 5 μm, 300 Å, PolyLC, Columbia, USA). The peptides were eluted at a flow rate of 200 μL/min with a linear gradient from 0 to 20% solvent B (25% v/v ACN, 500 mM ammonium formate) over 80 min, followed by a ramping up to 100% solvent B in 5 min and holding for 10 min. The peptides were detected at 214 nm absorbance and a total of 34 fractions were collected. Two independent iTRAQ experiments were conducted for the SPs and CWAPs samples.

Each SCX fraction was lyophilized in a speedvac and the resuspended in loading buffer (3% acetonitrile, 0.1% acetic acid, 0.01% TFA) and loaded onto a C18 capillary trap cartridge (LC Packings, USA) and then separated on a 15 cm nanoflow analytical C18 column (PepMap 75 μm id, 3 μm, 100 Å) at a flow rate of 200 nL/min on a Tempo nanoflow multidimensional LC system (ABsciex, USA). Solvent A composition was 3% ACN v/v, 0.1% acetic acid v/v; whereas solvent B was 97% ACN v/v, 0.1% acetic acid v/v. Peptides separation was performed with a linear gradient from 3 to 40% of solvent B for 2 h, followed by an increasing to 90% of solvent B in 10 min and hold for 10 min. Eluted peptides were introduced by ESI into a quadrupole time-of-flight mass spectrometer (QSTAR Elite MS/MS system, ABsciex Inc., USA). The source nebulizing gas and curtain gas were set at 12 and 20, respectively. Ion spray voltage was 2200V and the temperature was 80°C. The data were collected in the Information depended acquisition mode. A TOF-MS scan was done (m/z 300–1800, 0.25 s) automatically followed by the MS/MS scan (m/z 50–2000, 30–2000 ms) of the three abundant peptide ions per cycle. A 60 s exclusion was set for the former target ions. Automatic collision energy, automatic MS/MS accumulation and dynamic exclusion were selected in Analyst QS software.

PROTEIN IDENTIFICATION, QUANTIFICATION, AND FUNCTIONAL CLASSIFICATION

The identification and quantification of proteins were performed using ProteinPilot™ Software 4.0. The database was UniProt for maize (March 29, 2011; 84916 entries). The searching parameters were set as iTRAQ peptide label, cysteine alkylation with methyl methanethiosulfonate, trypsin digestion, identification focus for biological modifications and BIAS correction. The unused score threshold was set to >1.3 (equivalent to 95% confidence or better) and p-value < 0.05 to ensure that quantitation was based on at least three unique peptides. The proteins were considered for validation if they were significant in both independent experiments.

For functional classification, the protein lists were analyzed according to the Gen Ontology (GO) using the Singular Enrichment Analysis (SEA) tool (http://bioinfo.cau.edu.cn/agriGO/) (Du et al., 2010) for biological, molecular and cellular function classification, considering a background database list

of *Zea mays* and the Plant Slim algorism available in the agriGO. The results were exported directly to REVIGO (Supek et al., 2011) in order to visualize the clusters of the enriched GO terms. The KEGG encyclopedia was used for the identification of pathways (http://www.genome.jp/kegg) (Kanehisa et al., 2012).

RNA EXTRACTION, cDNA SYNTHESIS, AND QUANTITATIVE REAL-TIME PCR (qRT-PCR)

All RNA studies were done on the same sample papers as those used in the above iTRAQ analyses. RNA extraction, cDNA synthesis, and qRT-PCR were done as described previously (Chourey et al., 2010; LeClere et al., 2010). Briefly, total RNA was extracted from each sample of 100 mg tissue (a total of four samples, two for each genotype) and treated with DNA-free DNase I (Ambion, TX, USA). Purified RNA samples were quantified using a Nanodrop ND-4000 Spectrophotometer (Thermo Scientific, DE, USA) and evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). First-strand cDNA was synthesized with 5 μg of purified RNA using reverse transcriptase (RT) Superscript III (Invitrogen, CA, USA). qRT-PCR assays for each target were performed via MyiQ ver. 2.0 with iQ ver. 5.0 (Bio-Rad, CA, USA), using the primers (**Table S1**) and SYBER green dye method as previously described (Koh et al., 2010). Our previous studies have shown that absolute q-PCR method, as used here, is a reliable estimate of gene expression based on several genes and an excellent concordance between the low resolution Northern blot hybridization and absolute q-PCR values (Chourey et al., 2010; LeClere et al., 2010).

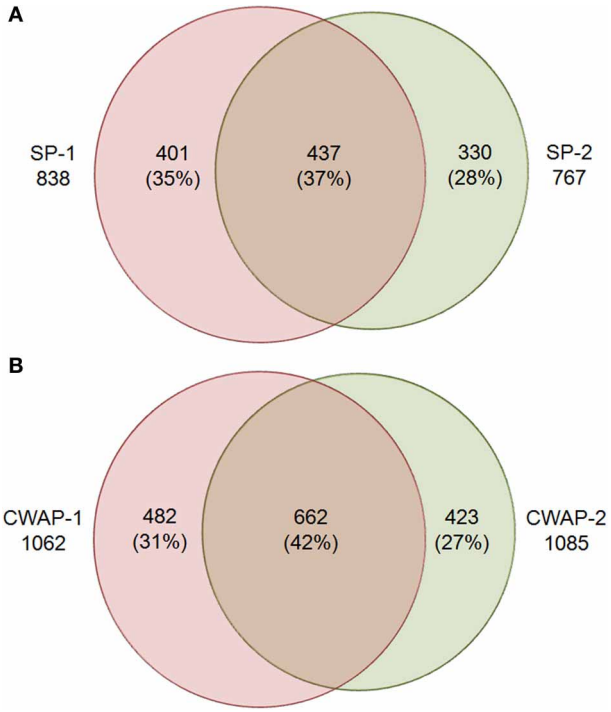

FIGURE 2 | Venn diagram showing the overlap of protein identities in two biological replicates for (A) soluble proteins (SPs), SPs-1 and SPs-2 are biological replicates; and (B) cell wall associated proteins (CWAPs) in two biological replicates.

RESULTS AND DISCUSSION

IDENTIFICATION OF SOLUBLE PROTEINS (SPs) AND CELL WALL ASSOCIATED PROTEINS (CWAPs) IN BETL ENRICHED REGIONS

One of the main functions of the basal part of maize endosperm is to acquire and transfer of nutrients and water from maternal plant to developing seed through the BETL localized in the basal region. The lack of *Mn*1-encoded INCW2 protein in the *mn*1 BETL has led to many pleiotropic changes in the mutant relative to the *Mn*1 (Chourey et al., 2012). The 12 DAP stage used in this study is ideal as it marks the highest levels of INCW2 activity (Cheng et al., 1996), and a major point of developmental and metabolic switch from cell division, cell elongation phase to the initiation of storage phase which is expected to be associated with proteomic changes in the *Mn*1 and the mutant *mn*1 endosperm. Here we have used a multiplex iTRAQ approach in the systematic identification and quantification of the expression levels of both soluble and CWAPs in the two genotypes, *Mn*1 and *mn*1. The experimental strategy based on the use of LC-MS/MS and the GO functional classification is depicted in the **Figure 1**. We identified a total of 1168 SPs in two independent experiments with a 1.3 unused score threshold (95% confidence) (**Table S2**). A total of 437 proteins were identified in both experiments representing a 38% overlap, and there were 401 (34%) and 330 (28%) unique proteins for experiments 1 and 2, respectively (**Figure 2A**). For CWAPs, there were 1567 identifications in two independent experiments (**Table S2**). A total of 662 proteins were identified in both experiments, representing a 42% overlap, and there were 482 (31%) and 423 (27%) unique proteins identified in experiments 1 and 2, respectively (**Figure 2B**). When comparing SPs and CWAPs protein identifications, only 36% of the IDs were shared between the two sample types, suggesting the complementary nature of the different extractions (**Tables S2**, **S3**). Reproducibility of protein identification is known to be affected in replicates of iTRAQ experiments, and any given run may identify only a subset of relevant peptides in a complex mixture causing variation among analytical runs (Lee and Koh, 2011; Owiti et al., 2011). The overlap showed for SPs, CWAPs, and in between the two independent replicate experiments was comparable to previous reports (Chen et al., 2007) that show the range of 25–40% in similar iTRAQ analyses.

FUNCTIONAL ANNOTATION OF IDENTIFIED PROTEINS

In order to understand the functions of the identified SPs and CWAPs and their related processes, a Gene Ontology (GO) classification was performed using the agriGO Single Enrichment Analysis (SEA) tool (http://bioinfo.cau.edu.cn/agriGO). A plant GO slim type was used for the SPs in which 984 (84%) of the 1168 proteins were annotated. The results showed 14 GO terms enriched for biological process, 5 GO terms for molecular function, and 17 GO terms for cellular component (**Table S4**). In general, the GO terms that were significantly enriched are related to cellular and metabolic processes. Specifically those belonging to primary and secondary metabolic process, catabolic process, carbohydrates metabolic process and cellular homeostasis (**Figure 3A**) that could be attributed to the INCW2-deficiency

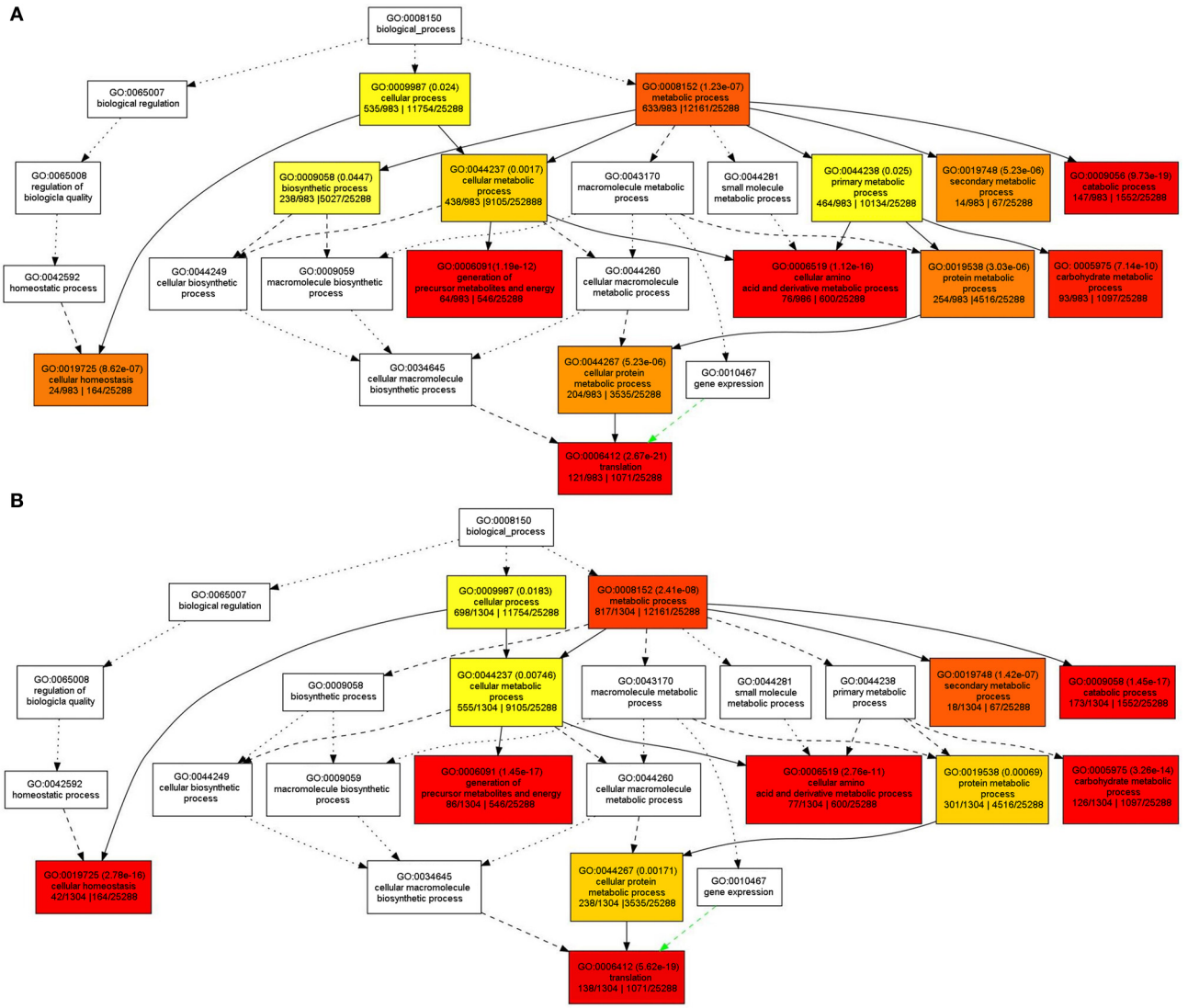

FIGURE 3 | GO classification of enriched biological functions in (A) SPs and (B) CWAPs with agriGO tool kit.

in the basal region of *mn*1 seeds. Following the same strategy as in the analysis of SPs, a total of 1034 (83%) entries of the 1567 CWAPs were annotated; i.e., 12 GO terms enriched for biological process, 5 GO terms for molecular function, and 18 GO terms for cellular component (**Table S5**). The terms for biosynthetic process and primary metabolic process were not found in the CWAPs (**Figure 3B**). Similar proteomic studies in developing grains of rice also show the predominant role of carbohydrate metabolic processes in seed development (Lee and Koh, 2011).

GO classification results are usually displayed as a tree form, in which if one of the end point of the branch is enriched, the previous nodes to the branch will be enriched as well, leading to some redundancy. The REVIGO tool was used to analyze the GO enriched terms in order to reduce the redundancy and allow visualization of the most informative common ancestor nodes. **Figure S1** shows the enriched biological functions in the SPs and CWAPs. Interestingly, both fractions are strikingly similar. With the exception of biological process and primary metabolic process in SPs, both analyses revealed the translation process as the main one affected by the mutation and directly related to the cellular homeostasis process (**Figures S1A,B**). These results suggest that multiple biological processes were affected in the *mn*1 mutant and the analysis of both fractions suggests a complementary nature of the two protein fractions in the basal endosperms.

DIFFERENTIALLY EXPRESSED PROTEINS IN mn1 MAIZE BASAL ENDOSPERM

Differential expression of proteins in any two independent experiments by iTRAQ is considered statistically significant if (i) they show an average change of 20% or greater, (ii) a *p-value* less than

Table 1 | Proteins identified as differentially expressed from iTRAQ LC-MS/MS of soluble proteins (SPs).

Accession	Name	Experiment 1		Experiment 2	
		*mn*1/*Mn*1	*SD*	*mn*1/*Mn*1	*SD*
B6SZ52	Putative uncharacterized protein	**0.35**	**0.04**	**0.42**	**0.02**
Q9SPJ9, Q9SPK0	[a]Cell wall invertase 2	**0.37**	**0.05**	**0.30**	**0.02**
B6EBQ2	Indole-3-acetic acid amido synthetase	**0.52**	**0.01**	**0.77**	**0.01**
B4G0K5	Putative uncharacterized protein	**0.58**	**0.01**	0.81	0.01
B6TBZ8	Alanine aminotransferase 2	**0.64**	**0.01**	0.85	0.02
B6TEC1	Sorbitol dehydrogenase	**0.67**	**0.00**	0.80	0.05
Q43706	Sus1 protein	**0.71**	**0.01**	0.93	0.03
Q548K3	Farnesyl diphosphate synthase	**0.74**	**0.02**	1.01	0.02
Q5EUE1	[b]Protein disulfide isomerase	**0.76**	**0.02**	0.96	0.02
B7ZXK1	Aspartate aminotransferase	**0.76**	**0.00**	0.92	0.01
E9NQE5	Pyruvate orthophosphate dikinase 1	**0.78**	**0.02**	0.90	0.03
B6T4Q5	Pro-resilin	0.91	0.00	**1.32**	**0.09**
Q6VWF6	O-methyltransferase (Fragment)	0.92	0.04	**1.20**	**0.02**
C0HHC4	Nucleoside diphosphate kinase	0.92	0.03	**1.26**	**0.05**
C4J473	Similar to oligopeptidase A-like from *Oryza sativa* (Q6K9T1)	0.93	0.01	**1.25**	**0.01**
B6TRW8	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	0.95	0.02	**1.32**	**0.00**
Q84TL6	[c]Legumin-like protein	0.96	0.01	**1.30**	**0.01**
F8UV61	Cell division cycle protein 48 (Fragment)	0.96	0.04	**1.23**	**0.03**
B6TNF1	Calnexin	0.98	0.08	**1.24**	**0.01**
C0P2V1	Similar to leucine aminopeptidase 2, chloroplastic from *Oryza sativa* (Q6K669)	0.98	0.05	**1.35**	**0.03**
C0HGV5	Enolase	1.01	0.02	**1.46**	**0.08**
Q5EUD6	[b]Protein disulfide isomerase	1.02	0.01	**1.32**	**0.04**
B7ZWY9	Citrate synthase	1.02	0.02	**1.35**	**0.03**
B4G0S0	Similar to UMP synthase from *Zea mays* (Q9LKI4)	1.03	0.07	**1.28**	**0.04**
Q6R987	ATP synthase subunit alpha	1.03	0.01	**1.23**	**0.04**
C0PL01	Similar to T-complex protein 1 subunit gamma from *Zea mays* (B6UCD0)	1.03	0.01	**1.30**	**0.01**
B6SXV4	Peroxisomal fatty acid beta-oxidation multifunctional protein	1.03	0.02	**1.28**	**0.04**
B6U0V6	Endoplasmin	1.03	0.02	**1.33**	**0.04**
B4FNM4	Similar to 60S acidic ribosomal protein P0 from *Zea mays* (O24573)	1.03	0.02	**1.42**	**0.00**
B4FH47	Similar to USP family protein from *Zea mays* (B6TC12)	1.04	0.05	**1.46**	**0.02**
Q8S4W9	Pyruvate decarboxylase	1.04	0.05	**1.23**	**0.04**
B4FFH8	Adenosine kinase 2	1.04	0.00	**1.31**	**0.02**
B6T856	L-lactate dehydrogenase	1.05	0.02	**1.35**	**0.02**
C4J5G3	Similar to dihydrolipoyl dehydrogenase from *Sorghum bicolor* (C5XIY9)	1.05	0.01	**1.34**	**0.00**
B4FGL3	Similar to mitochondrial ATP synthase from *Zea mays* (B6TCR9)	1.05	0.04	**1.39**	**0.01**
B6TDE0	NADP-dependent oxidoreductase P1	1.07	0.03	**1.42**	**0.04**
B6TJB6	Proteasome subunit alpha type	1.07	0.00	**1.39**	**0.06**
C4J4E4	Similar to cytosolic monodehydroascorbate reductase from *Oryza sativa* (Q9XFZ3)	1.08	0.04	**1.37**	**0.04**
B6TI78	Peptidyl-prolyl isomerase	1.08	0.05	**1.44**	**0.00**
Q5GAU1	Putative alanine aminotransferase	1.09	0.01	**1.35**	**0.00**
B6UHU1	Catalase	1.09	0.03	**1.26**	**0.00**
B6U4A3	Heat shock 70 kDa protein	1.10	0.02	**1.27**	**0.09**
B8A1R8	Similar to 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase from *Zea mays* (B6UF55)	1.11	0.03	**1.27**	**0.03**
B4G080	Caffeoyl-CoA O-methyltransferase 1	1.11	0.01	**1.87**	**0.07**
B4FCK0	USP family protein	1.11	0.01	**1.37**	**0.02**
B6T3G4	40S ribosomal protein S19	1.12	0.02	**1.45**	**0.04**

(Continued)

Table 1 | Continued

Accession	Name	Experiment 1		Experiment 2	
		mn1/Mn1	*SD*	*mn1/Mn1*	*SD*
B8A2W6	Putative uncharacterized protein	1.12	0.04	**1.55**	**0.01**
Q9LLB8	Exoglucanase	1.13	0.06	**1.41**	**0.03**
Q5EUD7	[b]Protein disulfide isomerase	1.14	0.03	**1.49**	**0.04**
C0PK05	Similar to lactoylglutathione lyase from *Zea mays* (B6TPH0)	1.17	0.02	**1.54**	**0.01**
C0P820	Similar to 3-ketoacyl-CoA thiolase 2, peroxisomal, putative, expressed from *Oryza sativa* (Q94LR9)	1.18	0.04	**1.38**	**0.03**
B6SXW8	RuBisCO large subunit-binding protein subunit alpha	**1.21**	**0.01**	**1.60**	**0.00**
B8A1M2	Similar to thioredoxin-like proteon 5 from *Zea mays* (B6TP19)	**1.22**	**0.01**	**1.79**	**0.06**
C0HF77	Putative uncharacterized protein	**1.22**	**0.05**	**1.35**	**0.01**
B6UAU9	Rhicadhesin receptor	**1.23**	**0.04**	**1.47**	**0.02**
B6UAK0	6-phosphogluconolactonase	**1.28**	**0.06**	**1.71**	**0.00**
Q8W2B6	UDP-glucosyltransferase BX9	**1.28**	**0.04**	**1.54**	**0.02**
Q84TL7	[c]Legumin-like protein	**1.28**	**0.01**	**1.50**	**0.03**
B6T522	40S ribosomal protein S14	**1.30**	**0.02**	**1.46**	**0.04**
B8A326	Similar to plasma membrane ATPase from *Oryza sativa* (Q7XPY2)	**1.33**	**0.03**	**1.60**	**0.04**
B6TVW2	Germin-like protein subfamily 1 member 17	**1.37**	**0.09**	**1.62**	**0.00**
B6T4C7	Peptidyl-prolyl cis-trans isomerase	**1.42**	**0.09**	**1.88**	**0.03**
C4J9Y2	Similar to stem-specific protein TSJT1 from *Zea mays* (B4FQW0)	**1.42**	**0.02**	**2.08**	**0.06**
C4J409	Putative uncharacterized protein	**1.44**	**0.01**	**1.87**	**0.09**
B8A3M0	[d]Glutamine synthetase	**1.45**	**0.07**	**1.86**	**0.06**
B4G218	Putative uncharacterized protein	**1.51**	**0.02**	**2.24**	**0.03**
B4FTP4	Putative uncharacterized protein	**1.59**	**0.06**	**2.12**	**0.05**
B4FT23	14-3-3-like protein	**1.63**	**0.05**	**2.09**	**0.10**
B4FG65	Similar to catalytic/hydrolase from *Zea mays* (B6U0A3)	**2.43**	**0.09**	**2.83**	**0.15**
B4FFQ0	Thioredoxin	**2.68**	**0.13**	**2.93**	**0.09**
B6SHX0	5a2 protein	**0.46**	**0.00**	ND	ND
Q43359	Cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC4	**0.62**	**0.04**	ND	ND
B4FK84	Similar to glutathione transferase III(A) from *Zea mays* (Q9ZP62)	**0.79**	**0.00**	ND	ND
B6TM55	[e]APx1-cytosolic ascorbate peroxidase	**1.23**	**0.04**	ND	ND
B4FFJ4	Similar to alpha-galactosidase from *Oryza sativa* (Q9FXT4)	**1.42**	**0.09**	ND	ND
B6TYM9	[f]Vignain	**1.61**	**0.04**	ND	ND
C5JA67	BETL-9 protein	ND	ND	**0.45**	**0.01**
B6TP93	Fructokinase-2	ND	ND	**1.20**	**0.02**
B4FBF4	Serine hydroxymethyltransferase	ND	ND	**1.24**	**0.01**
B6TJM5	26S protease regulatory subunit 6A	ND	ND	**1.26**	**0.02**
B4FZV6	Similar to 26S protease regulatory subunit 7 from *Oryza sativa* (Q9FXT9)	ND	ND	**1.26**	**0.00**
B6SJ21	Guanine nucleotide-binding protein beta subunit-like protein	ND	ND	**1.27**	**0.02**
B6TMX0	Pyruvate kinase	ND	ND	**1.32**	**0.07**
Q94G64	T-cytoplasm male sterility restorer factor 2	ND	ND	**1.34**	**0.02**
C0PHP3	Similar to 60 kDa chaperonin beta subunit from *Oryza sativa* (Q6ZFJ9)	ND	ND	**1.35**	**0.03**
B6T782	40S ribosomal protein SA	ND	ND	**1.36**	**0.03**
C0PGM6	Similar to 26S protease regulatory subunit S10B from *Zea mays* (B4FTV9)	ND	ND	**1.37**	**0.04**
B4FBY6	Similar to caffeoyl CoA 3-O-methyltransferase from *Zea mays* (Q7X6T0)	ND	ND	**1.40**	**0.01**
B4G1D2	CBS domain protein	ND	ND	**1.49**	**0.07**
B6SIF5	Translationally-controlled tumor protein	ND	ND	**1.49**	**0.03**
C0P397	Similar to adenine phosphoribosyltransferase 2 from *Zea mays* (B6TGM2)	ND	ND	**1.49**	**0.03**
B8A0Q6	Similar to succinate dehydrogenase flavoprotein subunit, mitochondrial from *Zea mays* (B6U124)	ND	ND	**1.51**	**0.04**

(Continued)

Table 1 | Continued

Accession	Name	Experiment 1		Experiment 2	
		*mn*1/*Mn*1	*SD*	*mn*1/*Mn*1	*SD*
B7ZZ39	Glutamate dehydrogenase	ND	ND	**1.52**	**0.04**
B6UB73	[e]APx1-cytosolic ascorbate peroxidase	ND	ND	**1.58**	**0.03**
B4G1X1	Putative uncharacterized protein	ND	ND	**1.59**	**0.05**
B6SS31	Putative uncharacterized protein	ND	ND	**1.60**	**0.03**
B9TSW1	[d]Glutamine synthetase	ND	ND	**1.61**	**0.04**
B7ZXW9	Similar to alpha-galactosidase from *Oryza sativa* (Q9FXT4)	ND	ND	**1.62**	**0.01**
E7DDV3	Peroxisomal-CoA synthetase	ND	ND	**1.95**	**0.06**
B6TLR1	[f]Vignain	ND	ND	**2.25**	**0.06**

Values shown in bold indicate protein fold changes lower than 0.8 or higher than 1.2 and p-value < 0.05.

Ratios mn1/Mn1 are average values of the two independent replicates, each quantified with at least three unique peptides.

[a]Isoform with a single amino acid substitution, quantification was done only with the common peptides.

[b,c,d,e,f]Isoforms detected in the experiment.

0.05, and (iii) obtained from measuring at least three unique peptides to ensure significant quantitative changes (Ali et al., 2010; Zhu et al., 2010). With these criteria, we retrieved a total of 100 SPs that were differentially expressed; among which 15 showed decreased and 85 showed increased levels in the mutant as compared to the *Mn*1 (**Table 1**). Among the CWAPs, a total of 91 proteins were differentially expressed, of which 33 showed decreased and 58 showed increased levels in the mutant as compared to the *Mn*1 (**Table 2**). Of the total 191 differentially expressed proteins in both SPs and CWAPs samples, only 15 proteins were common to both fractions (**Table S6**), suggesting that extraction protocol was rigorous in allowing most of the proteins to be fractionated. It should be noted that the salt extraction protocol was effective in releasing ionically bound CWAPs. All shared proteins exhibited similar expression ratios except for B4G0K5, which was decreased in the SPs and increase in the CWAPs. This protein has no known functions and no annotations could be found to assign or hypothesize its function. It is not surprising that certain CWAPs were also released into the soluble fraction during the homogenization in low salt concentration of buffer, as shown previously for the INCW2 (Carlson and Chourey, 1999).

Table 3 shows GO annotations for SPs that were differentially expressed in the two genotypes. It includes proteins in catabolic process, cellular homeostasis and carbohydrate metabolism as biological functions through SEA described in the Materials and Methods. The differentially expressed CWAPs did not show any significant enrichment for biological function (**Table 3**). In general, the main processes that are affected due to the lack of INCW2 are related to the compensation of the energy supply via the carbohydrate metabolic process and establishment of normal activity in the cell through the catabolic process. Similar proteomic study in developing rice grain shows that carbohydrate metabolic process, transport, and localization are the main cellular events (Lee and Koh, 2011).

The KEGG mapper (http://www.genome.jp/kedd/mapper.html) was used to investigate the metabolic pathways of proteins grouped in catabolic process, cellular homeostasis and carbohydrate metabolic process. As shown in **Table 3**, the catabolic process involves mainly proteins that are related to the biosynthesis of secondary metabolites, cellular homeostasis including enzymes related to protein processing in ER and carbohydrate metabolic process; and glycolysis and gluconeogenesis processes. For the catabolic process, increased levels of proteins were related to proteasome, as well as proteases such as 26S protease regulatory subunit 6A, mitochondrial processing peptidase, and K01365 cathepsin L. Proteome and transcriptome studies in developing seeds of *Medicago truncatula* show transcriptional up-regulation for proteins that may play a role in protein degradation either during reserve accumulation or maturation phase (Gallardo et al., 2007). Similar proteome analyses by (Méchin et al., 2007) reported an important function of turnover of proteins that was associated with the switch from growth and differentiation to storage in maize. In *mn*1 seeds, the presence of proteases in an early stage of development suggests important protein turnover and processing taking place in the BETL region. Interestingly, a leucine aminopeptidase, known to participate in the glutathione metabolism, was increased in the mutant. These data indicate a role of potential redox regulation mediated by glutathione in the BETL enriched basal endosperm.

The cellular homeostasis group was mainly composed of protein disulfide isomerases (PDIs), which showed increased ratios in the *mn*1 when compared with the *Mn*1. Genome-wide search has shown 22 PDI-like sequences in maize (Houston et al., 2005), and the PDIs are known to act as molecular chaperons that contain thioredoxin domains critical in the formation of proper disulfide bonds during protein folding. Thus, the increased abundance of PDI protein in the *mn*1 mutant was consistent with its proposed role in ER stress resulting from reduced Golgi in the BETL of the *mn*1 than the *Mn*1 (Kang et al., 2009). Interestingly, an uncharacterized protein with a thioredoxin motif (B4FFQ0) (**Table 3**) showed a 2.8-fold increase in *mn*1, suggesting an up-regulation of the gene essential in the folding of new proteins as well as in response to the oxidation/reduction generated by metabolic stress in the mutant lacking INCW2 (Freedman et al., 1994; Houston et al., 2005). These two groups of proteins may have complementary functions in response to the lack of glucose/fructose in the

Table 2 | Proteins identified as differentially expressed from iTRAQ LC-MS/MS of cell wall associated proteins (CWAPs).

Accession	Name	Experiment 1		Experiment 2	
		*mn*1/*Mn*1	*SD*	*mn*1/*Mn*1	*SD*
Q9SPK0	Cell wall invertase 2	**0.38**	**0.02**	**0.27**	**0.00**
Q19VG6	Major latex protein 22	**0.45**	**0.00**	**0.51**	**0.02**
Q4FZ46	Cystatin	**0.48**	**0.00**	**0.54**	**0.01**
B6T6D4	Peroxidase 27	**0.52**	**0.02**	**0.53**	**0.00**
C0P6F8	Similar to sucrose synthase 2 from *Zea mays* (P49036)	**0.57**	**0.00**	**0.71**	**0.01**
Q9FER8	HMGI/Y protein	**0.57**	**0.01**	**0.44**	**0.01**
B4FSS8	Putative uncharacterized protein	**0.57**	**0.03**	**0.62**	**0.00**
B4G004	Similar to beta-glucosidase 7 from *Oryza sativa* (Q75I93)	**0.63**	**0.04**	**0.60**	**0.04**
C4JC17	Putative uncharacterized protein	**0.63**	**0.09**	**0.66**	**0.00**
B6TDW7	Secretory protein	**0.66**	**0.03**	**0.59**	**0.02**
B6T4G7	Fibrillarin-2	**0.68**	**0.02**	0.82	0.00
B6TPA2	Putative uncharacterized protein	**0.68**	**0.01**	**0.57**	**0.00**
B4FHA8	Similar to histone deacetylase HDT2 from *Zea mays* (Q9M4U5)	**0.69**	**0.03**	**0.76**	**0.12**
Q4FZ49	Putative cystatin	**0.69**	**0.02**	**0.75**	**0.00**
B7ZXK1	Aspartate aminotransferase	**0.73**	**0.01**	**0.75**	**0.02**
C0P8K0	Similar to 3-hydroxybutyryl-CoA dehydratase from *Zea mays* (B6TF44)	**0.75**	**0.00**	**0.77**	**0.05**
B6TKT8	Esterase	**0.76**	**0.02**	**0.72**	**0.03**
B6TS72	U2 small nuclear ribonucleoprotein A	**0.76**	**0.03**	**0.79**	**0.02**
B4FT31	Similar to chloride intracellular channel 6 from *Zea mays* (B6UCX2)	**0.76**	**0.02**	**0.72**	**0.02**
E9NQE5	Pyruvate orthophosphate dikinase 1	**0.77**	**0.02**	**0.74**	**0.00**
B6TIQ8	ATP/GTP binding protein	**0.78**	**0.00**	0.85	0.05
B6TE01	Myosin-like protein	0.80	0.07	**0.76**	**0.01**
B6THU9	Peroxidase 39	0.81	0.01	**0.76**	**0.00**
B6SLX1	Chaperonin	0.85	0.04	**0.79**	**0.02**
B6UDP8	Protein CYPRO4	0.97	0.01	**0.76**	**0.01**
B6TG70	Mitochondrial-processing peptidase beta subunit	1.01	0.01	**1.28**	**0.02**
Q9SAZ6	Phosphoenolpyruvate carboxylase	1.10	0.06	**1.20**	**0.02**
B4FER8	Acyl-CoA-binding protein	1.13	0.03	**1.23**	**0.05**
B4FNM4	Similar to 60S acidic ribosomal protein P0 from *Zea mays* (O24573)	1.18	0.03	**1.35**	**0.01**
B6TRV8	Eukaryotic initiation factor 5C CG2922-PF, isoform F	**1.20**	**0.03**	1.19	0.06
C0P8C6	Similar to TCP-1/cpn60 chaperonin from *Oryza sativa* (C6F1N7)	**1.20**	**0.12**	1.10	0.02
Q43712	Calcium-binding protein	**1.20**	**0.01**	**1.32**	**0.03**
B8A0J2	Putative uncharacterized protein	**1.21**	**0.02**	1.13	0.02
B6SPX4	Tubulin alpha-3 chain	**1.22**	**0.02**	1.15	0.03
C0P2V1	Similar to leucine aminopeptidase 2, chloroplastic from *Oryza sativa* (Q6K669)	**1.23**	**0.02**	1.03	0.06
B4FTP4	Putative uncharacterized protein	**1.26**	**0.01**	**1.65**	**0.05**
C0PDB6	Similar to 3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase from *Zea mays* (B6TJ78)	**1.27**	**0.00**	**1.39**	**0.08**
Q9AXG8	Lipoxygenase	**1.28**	**0.01**	1.11	0.00
Q9M588	Prohibitin	**1.30**	**0.02**	**1.21**	**0.08**
B6TB97	40S ribosomal protein S3	**1.31**	**0.00**	**1.25**	**0.02**
B2ZAF9	Malate dehydrogenase	**1.31**	**0.04**	**1.22**	**0.02**
C0P4D8	Similar to dynamin-2A from *Zea mays* (B6UEQ3)	**1.32**	**0.03**	**1.30**	**0.04**
C0PH85	Similar to tubulin beta-3 chain from *Eleusine indica* (Q9ZPN8)	**1.32**	**0.01**	**1.33**	**0.04**
C0P406	Putative uncharacterized protein	**1.33**	**0.07**	1.12	0.04
Q6R9G1	NADH dehydrogenase subunit 7	**1.33**	**0.02**	1.13	0.03
Q8S4W9	Pyruvate decarboxylase	**1.33**	**0.02**	1.13	0.00
B6TYX3	USP family protein	**1.35**	**0.04**	**1.25**	**0.02**
C0PHK6	Similar to dynamin-related protein 1C from *Zea mays* (B6U1C4)	**1.38**	**0.09**	**1.80**	**0.22**
C0HFU7	Phospholipase D	**1.38**	**0.05**	**1.35**	**0.08**
Q5EUD5	Protein disulfide isomerase	**1.39**	**0.04**	**1.23**	**0.01**
B4FV87	Jasmonate-induced protein	**1.45**	**0.03**	**1.39**	**0.03**

(Continued)

Table 2 | Continued

Accession	Name	Experiment 1		Experiment 2	
		*mn*1/*Mn*1	*SD*	*mn*1/*Mn*1	*SD*
B4FUE0	GTP-binding protein PTD004	**1.45**	**0.02**	**1.40**	**0.02**
B4FCQ4	Cytochrome c oxidase subunit	**1.46**	**0.02**	**1.32**	**0.02**
C0PHF3	Similar to alpha-glucosidase like protein from *Hordeum vulgare* (B5U8Z1)	**1.47**	**0.05**	1.17	0.03
B4G0K5	Putative uncharacterized protein	**1.48**	**0.03**	1.19	0.05
B6U0V6	Endoplasmin	**1.52**	**0.00**	**1.36**	**0.02**
B6TC70	Acid phosphatase	**1.52**	**0.03**	**1.86**	**0.11**
B4F9U9	Putative uncharacterized protein	**1.59**	**0.01**	**1.44**	**0.00**
B6U4J8	Dynamin-related protein 1A	**1.60**	**0.00**	1.19	0.04
B4FX40	Similar to cysteine proteinase 1 from *Zea mays* (Q10716)	**1.62**	**0.01**	**1.56**	**0.02**
Q9M585	Stomatin-like protein	**1.64**	**0.01**	1.19	0.04
C4J409	Putative uncharacterized protein	**1.67**	**0.04**	**1.90**	**0.21**
C0HF77	Putative uncharacterized protein	**1.69**	**0.04**	**1.87**	**0.09**
B6T887	Salt tolerance protein	**1.76**	**0.09**	**1.81**	**0.01**
B4FKH7	Cytochrome b5	**2.03**	**0.06**	**1.75**	**0.05**
B4FSK9	Peroxidase 1	**2.23**	**0.13**	**1.48**	**0.04**
B6SHX0	5a2 protein	**0.53**	**0.02**	ND	ND
Q948J8	Uncleaved legumin-1	**0.63**	**0.02**	ND	ND
B4FSU9	Hydrolase, hydrolyzing O-glycosyl compounds	**0.68**	**0.01**	ND	ND
B4FP25	[a]40S ribosomal protein S19	**0.70**	**0.01**	ND	ND
C0PDM0	Similar to vaculor H+-pyrophosphatase from *Sorghum bicolor* (D9IG65)	**0.72**	**0.03**	ND	ND
C0P5P9	Glycylpeptide N-tetradecanoyltransferase	**0.78**	**0.02**	ND	ND
O49010	Herbicide safener binding protein	**0.78**	**0.04**	ND	ND
B6UCD0	T-complex protein 1 subunit gamma	**1.26**	**0.04**	ND	ND
B8A2Z3	Coatomer subunit gamma	**1.28**	**0.05**	ND	ND
C0P531	Similar to 26S proteasome non-ATPase regulatory subunit 3 from *Zea mays* (B6TBG8)	**1.34**	**0.05**	ND	ND
C0P5E7	Putative uncharacterized protein	**1.34**	**0.04**	ND	ND
B4G1Q6	Similar to protein Z from *Zea mays* (B6TS23)	**1.37**	**0.02**	ND	ND
B4FQK5	Eukaryotic peptide chain release factor subunit 1-1	**1.47**	**0.01**	ND	ND
C0PJ26	Similar to DEAD-box ATP-dependent RNA helicase 15 from *Oryza sativa* (Q5JK84)	**1.63**	**0.05**	ND	ND
B4FUK7	Putative uncharacterized protein	**2.04**	**0.08**	ND	ND
B4FG65	Similar to catalytic/hydrolase from *Zea mays* (B6U0A3)	**2.42**	**0.04**	ND	ND
C5JA67	BETL-9 protein	ND	ND	**0.49**	**0.00**
Q946V2	Legumin 1	ND	ND	**0.60**	**0.01**
B6T3G4	[a]40S ribosomal protein S19	ND	ND	**0.69**	**0.01**
B4FGC8	40S ribosomal protein S12	ND	ND	**0.75**	**0.00**
B4F7T9	Putative uncharacterized protein	ND	ND	**0.75**	**0.01**
C0PP73	Similar to ADP-ribosylation factor from *Triticum aestivum* (Q76ME3)	ND	ND	**1.21**	**0.00**
B4G178	ADP, ATP carrier protein	ND	ND	**1.27**	**0.09**
B4FRG8	Similar to mitochondrial 2-oxoglutarate/malate carrier protein from *Zea mays* (B6T8M6)	ND	ND	**1.30**	**0.01**
O24449	Translational initiation factor eIF-4A	ND	ND	**1.56**	**0.02**

Values shown in bold indicate protein fold changes lower than 0.8 or higher than 1.2 and p-value < 0.05.

Ratios mn1/Mn1 are average values of the two independent replicates, each quantified with at least three unique peptides.

[a]*Isoforms found in the experiment.*

*mn*1 basal endosperm, described previously (LeClere et al., 2010; Chourey et al., 2012).

Carbohydrate metabolism and glycolysis process are highly regulated in seeds. For examples, Gallardo et al. (2007) reported in *M. truncatula* seeds, the genes involved in glycolysis are differentially expressed in seed development, meanwhile those involved in starch synthesis are transiently expressed in seed filling. In the case of the carbohydrate metabolic process group, cell wall invertase 2 was reduced in the *mn*1, as expected. In addition, Sus1 protein was also decreased in the *mn*1. Although the conversion of sucrose to hexose via SUS uses half of the ATP in comparison to the invertase route, the SUS reaction is reversible and is sensitive to the hexose phosphate levels (Barratt et al., 2009), suggesting a possible connection between SUS and invertase in

Table 3 | Differentially expressed SPs with their predicted biological functions.

Biological process	Accession	Name	*mn*1/*Mn*1	*SD*	KEGG identifier	Pathway identifier
Catabolic process (GO:0009056)	C4J473	Putative uncharacterized protein	1.09	0.23	NF	NF
	C0P2V1	pco101682(581); LOC100381923; K01255 leucyl aminopeptidase [EC:3.4.11.1]	1.17	0.26	zma:100381923	zma00480, zma01100
	B6UHU1	Catalase isozyme B; K03781 catalase [EC:1.11.1.6]	1.17	0.12	zma:100857004	zma00380, zma00630, zma01100, zma01110, zma04146
	B7ZWY9	Putative uncharacterized protein (Pfam: citrate synthase domain)	1.18	0.23	EC 2.3.3.1	zma00020, zma00630, zma01100, zma01110
	B6T856	cl2158_1; LOC100282503 (EC:1.1.1.27); K00016 L-lactate dehydrogenase [EC:1.1.1.27]	1.2	0.22	zma:100282503	zma00010, zma00270, zma00620, zma00640, zma01100, zma01110
	B6TJB6	Proteasome subunit alpha type 5	1.23	0.22	zma:100283546	zma03050
	C0HGV5	Putative uncharacterized protein (Pfam: enolase domain)	1.24	0.31	NF	NF
	B6TJM5	26S protease regulatory subunit 6A	1.26	0.02	NF	NF
	B4FZV6	Putative uncharacterized protein	1.26	0	NF	NF
	C0HF77	Uncharacterized LOC100304374	1.29	0.09	zma:100304374	NF
	B6TRW8	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	1.32	0	zma:100284269	zma00020, zma00310, zma01100, zma01110
	B6TMX0	Pyruvate kinase, cytosolic isozyme (EC:2.7.1.40)	1.32	0.07	zma:100283899	zma00010, zma00230, zma00620, zma00710, zma01100, zma01110
	B8A2W6	pco071606; LOC100280280; K01412 mitochondrial processing peptidase [EC:3.4.24.64]	1.34	0.31	zma:100280280	NF
	C0PGM6	Uncharacterized LOC100383510; K03064 26S proteasome regulatory subunit T4	1.37	0.04	zma:100383510	zma03050
	B6UAK0	umc2374; LOC100285843 (EC:3.1.1.31); K01057 6-phosphogluconolactonase [EC:3.1.1.31]	1.49	0.3	zma:100285843	zma00030, zma01100, zma01110
	B8A0Q6	TIDP3457; LOC100279930; K00234 succinate dehydrogenase (ubiquinone) flavoprotein subunit [EC:1.3.5.1]	1.51	0.04	zma:100279930	zma00020, zma00190, zma01100, zma01110
	B6TYM9	cl13598_-2; LOC100284911; K01365 cathepsin L [EC:3.4.22.15]	1.61	0.04	zma:100284911	zma04145
	B6TLR1	Vignain	2.25	0.06	NF	NF
Cellular homeostasis (GO:0019725)	Q5EUE1	PDIL1-1; protein disulfide isomerase; K09580 protein disulfide-isomerase A1 [EC:5.3.4.1]	0.86	0.14	zma:606409	zma04141
	Q5EUD6	pdi7, PDIL2-2; protein disulfide isomerase7; K09584 protein disulfide-isomerase A6 [EC:5.3.4.1]	1.17	0.22	zma:606414	zma04141
	C4J5G3	Uncharacterized LOC100501719; K00382 dihydrolipoamide dehydrogenase [EC:1.8.1.4]	1.19	0.21	zma:100501719	zma00010, zma00020, zma00260, zma00280, zma00620, zma01100, zma01110

(Continued)

Table 3 | Continued

Biological process	Accession	Name	*mn*1/*Mn*1	*SD*	KEGG identifier	Pathway identifier
	Q5EUD7	pdi6, PDIL2-1; protein disulfide isomerase6; K01829 protein disulfide-isomerase [EC:5.3.4.1]	1.32	0.25	zma:606413	zma04141
	B4FFQ0	Uncharacterized LOC100193620 T(Pfam: thioredoxin motif)	2.8	0.18	zma:100193620	NF
Carbohydrate metabolic process (GO:0005975)	Q9SPJ9, Q9SPK0	Cell wall invertase 2	0.33	0.05	EC 3.2.1.26	zma00052, zma00500, zma01100
	Q43359	TIDP3317, gpc4; LOC100037774; K00134 glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]	0.62	0.04	zma:100037774	zma00010, zma01100, zma01110
	Q43706	Sus1 protein	0.82	0.16	EC 2.4.1.13	zma00500, zma01100
	B7ZWY9	Putative uncharacterized protein (Pfam: citrate synthase domain)	1.18	0.23	EC 2.3.3.1	zma00020, zma01100, zma01110, zma00630
	B6T856	cl2158_1; LOC100282503 (EC:1.1.1.27); K00016 L-lactate dehydrogenase [EC:1.1.1.27]	1.2	0.22	zma:100282503	zma00010, zma00270, zma00620, zma00640, zma01100, zma01110
	C0HGV5	Putative uncharacterized protein (Pfam: enolase domain)	1.24	0.31	NF	NF
	Q9LLB8	Exoglucanase	1.27	0.2	EC 3.2.1.91	NF
	B6TMX0	Pyruvate kinase, cytosolic isozyme (EC:2.7.1.40); K00873 pyruvate kinase [EC:2.7.1.40]	1.32	0.07	zma:100283899	zma00010, zma00230, zma00620, zma00710, zma01100, zma01110
	B4FFJ4	Similar to alpha-galactosidase from *Oryza sativa* (Q9FXT4)	1.42	0.09	EC 3.2.1.22	zma00052, zma0056, zma00600, zma00603
	B6UAK0	umc2374; LOC100285843 (EC:3.1.1.31); K01057 6-phosphogluconolactonase [EC:3.1.1.31]	1.49	0.3	zma:100285843	zma00030, zma01100, zma01110
	B4G1X1	Uncharacterized LOC100274554; K07407 alpha-galactosidase [EC:3.2.1.22]	1.59	0.05	zma:100274554	zma00561, zma00600, zma00603

KEGG pathway identifiers: zma00010:glycolysis/gluconeogenesis; zma00020:citrate cycle (TCA cycle); zma00030:pentose phosphate pathway; zma00052:galactose metabolism; zma00190:oxidative phosphorylation; zma00230:purine metabolism; zma00260:glycine, serine, and threonine metabolism; zma00270:cysteine and methionine metabolism; zma00280:valine, leucine, and isoleucine degradation; zma00310:lysine degradation; zma00380:tryptophan metabolism; zma00480:glutathione metabolism; zma00500:starch and sucrose metabolism; zma00561:glycerolipid metabolism; zma00600:sphingolipid metabolism; zma00603:glycosphingolipid biosynthesis; zma00620:pyruvate metabolism; zma00630:glyoxylate and dicarboxylate metabolism; zma00640:propanoate metabolism; zma00710:carbon fixation in photosynthetic organisms; zma01100:metabolic pathways; zma01110:biosynthesis of secondary metabolites; zma03050:proteasome; zma04141:protein processing in endoplasmic reticulum; zma04145:phagosome; zma04146:peroxisome.

maize BETL region. Physiological roles of these two sucrolytical enzymes are well studied in the transfer cells of developing seeds in cotton (Ruan et al., 2003; Pugh et al., 2010) and maize (Kang et al., 2009). The cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPDH in the *mn*1 was reduced by one half, suggesting low flux into the glycolysis pathway due to the lack of glucose/fructose in the BETL region (LeClere et al., 2010; Chourey et al., 2012). Another level of regulation can be related to the thioredoxin-like protein. Reoxidation of glyceroldehyde-3-phosphate dehydrogenase through oxidized thioredoxin seems to be a conserved mechanism for protecting the cells from oxidative stress. GAPDH catalyzes the reversible oxidative phosphorylation of glyceraldehide-3-phospate to 1, 3-biphosphoglycerate. A reduction in its activity results in the accumulation of dihydroxyacetone phosphate, which triggers the repression of glycolysis-related enzymes and certain enzymes of the Pentose Phosphate pathway (PPP) are induced as well. PPP enzymes are critical in the maintenance of cytoplasmic NADPH necessary for the antioxidant systems such as glutathione and thioredoxin (Ralser et al., 2007; Neilson et al., 2011). In plants, the GAPDH enzyme is inactivated to stop consumption of ATP for starch synthesis, and starch hydrolysis takes place for producing fuel for the plants (Nelson and Cox, 2008). A 6-phosphogluconolactonase like protein [EC: 3.1.1.31] (B6UAK0) (**Table 3**), known to participate in the PPP according to the KEGG, result was increased, supporting the hypothesis of protection from oxidative stress in the *mn*1 kernel.

SECRETORY PROTEINS IN THE BETL

As indicated previously, one of the main functions of the basal part of maize endosperm is acquisition and transfer of nutrition and water from maternal plant to developing seed. As in all transfer cells, the main feature of the BETL is the labyrinth-like WIG that increases the plasma membrane area comprised of cellulosic molecules, and enhances transport capacity in these cells (Pugh et al., 2010). In maize, these cells are particularly rich in endoplasmic reticulum (ER), Golgi, mitochondria, and secretory functions. In fact, many of these functions are greatly altered in the *mn*1 mutant due to the loss of the INCW2 (Kang et al., 2009). Therefore, we focused here in the identification of some of the proteins that showed the highest probability of localization in the secretory pathway (**Table 4**). Not surprisingly, the *Mn*1-encoded INCW2 was found with the highest score as it has an N-terminal secretory signal peptide (Taliercio et al., 1999). The results of the immunogold labeling of the ER and the secretory organelles are consistent with the notion that INCW2 is synthesized by ER-bound ribosomes and delivered to the WIG via the Golgi and TGN compartments (Kang et al., 2009).

Of the 37 secretory proteins listed in the **Table 4**, only the INCW2 and the BETL9 are previously reported to be associated

Table 4 | Proteins predicted to have a signal peptide for the secretory pathway using TargetP.

Accession	Name	SPs		CWAPs		Len	SP	Loc	RC
		*mn*1/*Mn*1	*SD*	*mn*1/*Mn*1	*SD*				
Q9SPK0	Cell wall invertase 2	0.33	0.05	0.33	0.08	592	0.99	S	1
C0HF77	Putative uncharacterized protein	1.29	0.09	1.78	0.12	778	0.95	S	1
B6U0V6	Endoplasmin	1.18	0.21	1.44	0.11	807	0.90	S	1
B6SHX0	5a2 protein	0.46	0.00	0.53	0.02	107	0.86	S	2
C5JA67	BETL-9 protein	0.45	0.01	0.49	0.00	107	0.74	S	2
B4FG65	Similar to catalytic/hydrolase from *Zea mays* (B6U0A3)	2.63	0.28	2.42	0.04	360	0.69	S	3
Q5EUD7	Protein disulfide isomerase	1.32	0.25	ND	ND	367	1.00	S	1
Q5EUE1	Protein disulfide isomerase	0.86	0.14	ND	ND	514	0.99	S	1
B6TNF1	Calnexin	1.11	0.18	ND	ND	534	0.99	S	1
Q5EUD6	Protein disulfide isomerase	1.17	0.22	ND	ND	366	0.99	S	2
B6TYM9	Vignain	1.61	0.04	ND	ND	376	0.99	S	1
B6UAU9	Rhicadhesin receptor	1.35	0.17	ND	ND	218	0.95	S	1
B4FFJ4	Similar to alpha-galactosidase from *Oryza sativa* (Q9FXT4)	1.42	0.09	ND	ND	423	0.95	S	1
B4G218	Putative uncharacterized protein	1.87	0.52	ND	ND	353	0.94	S	1
B4G1X1	Putative uncharacterized protein	1.59	0.05	ND	ND	423	0.94	S	1
B6TVW2	Germin-like protein subfamily 1 member 17	1.49	0.18	ND	ND	226	0.94	S	1
Q9LLB8	Exoglucanase	1.27	0.20	ND	ND	622	0.81	S	2
B6TLR1	Vignain	2.25	0.06	ND	ND	377	0.78	S	2
Q43712	Calcium-binding protein	ND	ND	1.26	0.09	421	1.00	S	1
B6TDW7	Secretory protein	ND	ND	0.63	0.04	228	0.99	S	1
C0PDM0	Similar to vaculor H+-pyrophosphatase from *Sorghum bicolor* (D9IG65)	ND	ND	0.72	0.03	762	0.99	S	1
B4FSK9	Peroxidase 1	ND	ND	1.85	0.53	362	0.97	S	1
B4FSU9	Hydrolase, hydrolyzing O-glycosyl compounds	ND	ND	0.68	0.01	576	0.96	S	1
Q4FZ46	Cystatin	ND	ND	0.51	0.05	127	0.95	S	1
Q4FZ49	Putative cystatin	ND	ND	0.72	0.05	129	0.95	S	3
B4FX40	Similar to cysteine proteinase 1 from *Zea mays* (Q10716)	ND	ND	1.59	0.04	371	0.94	S	1
B8A0J2	Putative uncharacterized protein	ND	ND	1.17	0.06	897	0.93	S	1
B6T6D4	Peroxidase 27	ND	ND	0.53	0.01	355	0.91	S	3
Q9M588	Prohibitin	ND	ND	1.25	0.07	284	0.89	S	1
Q946V2	Legumin 1	ND	ND	0.60	0.01	483	0.81	S	2
Q5EUD5	Protein disulfide isomerase	ND	ND	1.31	0.12	439	0.80	S	2
Q948J8	Uncleaved legumin-1	ND	ND	0.63	0.02	482	0.77	S	3
B6THU9	Peroxidase 39	ND	ND	0.78	0.03	328	0.77	S	4
B4G004	Similar to beta-glucosidase 7 from *Oryza sativa* (Q75I93)	ND	ND	0.62	0.02	502	0.76	S	3
B6TPA2	Putative uncharacterized protein	ND	ND	0.63	0.08	390	0.67	S	3
B6TC70	Acid phosphatase	ND	ND	1.69	0.24	524	0.57	S	4
B4F7T9	Putative uncharacterized protein	ND	ND	0.75	0.01	354	0.28	S	5

Table 5 | A comparative profile of RNA and protein levels of select proteins in the *Mn*1 and *mn*1 basal endosperms.

Gene number	Accession number	Genes		*AV*	*SD*	ρ	qPCR ratio *mn*1/*Mn*1	iTRAQ ratio *mn*1/*Mn*1
1	Q9SPK0	*Mn* 1	*Mn*1	4305	235	<0.01	0.01	0.39
			*mn*1	29	4			
2	Q41779	*BETL* 1	*Mn*1	35752	4649	<0.01	0.12	ND
			*mn*1	4229	6			
3	Q4FZ46	*Psei* 8	*Mn*1	999	62	<0.01	0.15	0.48
			*mn*1	151	8			
4	C5JA67	*BETL* 9	*Mn*1	34925	7122	<0.01	0.21	0.49
			*mn*1	7471	22			
5	B6SHX0	*5a* 2	*Mn*1	17863	5116	<0.01	0.11	0.55
			*mn*1	2047	152			
6	B6TDW7	*Secretory protein*	*Mn*1	537	34	<0.01	0.13	0.58
			*mn*1	69	1			
7	Q946V2	*Legumin*	*Mn*1	24393	2409	<0.01	0.56	0.59
			*mn*1	13729	925			
8	Q5EUE1	*PDI* 1	*Mn*1	10797	841	<0.01	0.53	0.75
			*mn*1	5689	1102			
9	C0PLF0	*PDI* 2	*Mn*1	891	27	<0.01	0.43	0.83
			*mn*1	379	52			
10	C0P5R8	*Amidase*	*Mn*1	508	59	<0.01	0.28	0.85
			*mn*1	140	8			
11	B6TT94	*Hypothetical protein*	*Mn*1	2016	199	<0.01	0.89	0.85
			*mn*1	1795	148			
12	B4F861	*IAA-amino acid hydrolase*	*Mn*1	1021	131	<0.01	0.93	0.88
			*mn*1	948	128			
13	C0HFV7	*Apyrase*	*Mn*1	1972	148	<0.01	0.29	1.18
			*mn*1	572	46			
14	B8A0J2	*Hypothetical protein*	*Mn*1	1668	87	<0.01	0.66	1.23
			*mn*1	1097	99			
15	Q9M588	*Prohibitin* 2	*Mn*1	1131	12	<0.01	0.20	1.28
			*mn*1	230	4			
16	B6U0V6	*Endoplasmin*	*Mn*1	729	126	≦0.05	0.70	1.51
			*mn*1	507	5			
17	Q43712	*Calcium-binding protein*	*Mn*1	4707	619	<0.01	0.46	1.20
			*mn*1	2160	81			
18	Q5EUD5	*PDI* 8	*Mn*1	2084	290	<0.01	0.60	1.36
			*mn*1	1258	25			
19	B4FX40	*Cysteine proteinase* 1	*Mn*1	8206	369	<0.01	0.73	1.61
			*mn*1	6027	244			
20	C0HF77	*Hypothetical protein*	*Mn*1	120	20	≧0.01	2.46	1.72
			*mn*1	295	44			

Data are presented as the mean of ±SD of six q-PCR analyses (three technical replicates of two biological samples).
AV, represents average # transcripts/ng total RNA.

with the BETL. Although the BETL9 is expressed abundantly at the RNA level (Gómez et al., 2009 and **Table 5**) and was greatly reduced in the *mn*1 at both RNA (Xiong et al., 2011) and protein levels, nothing is known about its function in seed development. Calnexin (B6TNF1), endoplasmin (B6U0V6), calreticulin (Q43712), PDIL1-1 (Q5EUE1), PDI7 (Q5EUD6), PDI8 (Q5EUD5), and a putative uncharacterized protein with a domain of HSP70 (B8A0J2) are actively involved in the protein processing of ER pathway. There were some proteins that participate in the protein recognition by the luminal chaperones, lectin related proteins and terminal misfolded targeting protein to the proteasome (**Table S7**). Prohibitin (Q9M588) was identified with high probability of localizing to the secretory pathway, with ratios of 1.28 and 1.32 in the two experiments. This protein is predicted to function in mitochondrial processes including protein processing, respiratory chain function and mitochondrial DNA organization (Van Aken et al., 2010). Cystatins (Q4FZ46) are proteins that inhibit cysteine proteases by binding of cystatins, and are believed to be involved in multiple functions, including biotic and abiotic stress tolerance, programed cell death, and

the regulation of various metabolic processes through processing and degradation of various proteins (Massonneau et al., 2005). Recently, van der Linde et al. (2012) demonstrated that cystatin suppresses host immunity by inhibiting apoplastic cysteine proteases that play a pivotal role in defense signaling in maize. Clearly, detailed functional characterization of these novel secretory proteins in basal endosperms is an exciting future direction of research.

RNA AND PROTEIN ABUNDANCE OF SELECT PROTEINS IN THE BASAL ENDOSPERM

Our previous studies have established that the *Mn*1 encoded INCW2 as specific biomarker in the BETL, and its expression is greatly reduced in the *mn*1 mutant (Cheng et al., 1996; Chourey et al., 2006). Our proteomic data here (**Tables 1**, **2**) are consistent with the previous results. Transcriptome analyses of the BETL led to a large catalog of genes that showed transcript abundance in the *Mn*1 basal endosperm (Xiong et al., 2011). **Table 5** shows results from a comparative analysis of expression of a few select genes at RNA level by q-PCR and by iTRAQ (**Table 1**) in these two genotypes. There was a qualitative concordance between RNA and the cognate protein levels for the first 10 of the 20 genes that showed *mn*1 to *Mn*1 ratios of <1.0. The ratios between *mn*1:*Mn*1 at both levels of expression were similar, <1.0, but the quantitative values were divergent. In contrast, the expression levels of the other seven genes (13–19) were highly divergent. The qPCR values were <1.0, but the iTRAQ ratios were >1.0. Only one gene (#20) showed a higher ratio in qPCR results than in the iTRAQ results. Two genes, #11 and #12, showed similar ratios of transcript and protein levels in the two genotypes. Overall, our data showed a high level of non-concordance between RNA and protein level expression. Such discrepancy is also described previously; Hajduch et al. (2010) showed an overall concordance of only 56% between RNA and protein levels in developmental seeds of Arabidopsis. It is clear that changes in RNA abundance may not reflect protein level changes due to posttranscriptional and posttranslational regulatory processes in the cells (Gallardo et al., 2007; Lan et al., 2011).

CONCLUSIONS

Transfer cells are ubiquitous in plants, fungi, and animal cells, and are well recognized for their role in solute acquisition and transport functions. Emerging evidence suggests that BETL plays a critical role in seed development (Kang et al., 2009; Pugh et al., 2010; Costa et al., 2012). Transcriptomic analyses in maize BETL identified important genes related to mitochondrial and stress related functions (Xiong et al., 2011). As shown in this study, transcriptomic changes do not directly translate into the changes and functions of proteins, which determine phenotypes. Here we provide information at the proteome level on the proteins and processes in BETL enriched tissues that are targets of the *Mn*1 mutation.

Using iTRAQ LC-MS/MS, we have identified over 2500 proteins and more than 130 differential proteins in the BETL enriched basal endosperm of *mn*1. A large array of diverse functions, including energy and carbohydrate metabolism, secondary metabolism and defense related processes were revealed. Highly regulated protein folding/degradation and redox control may function to maintain the cellular homeostasis. Furthermore, the levels of many secretory proteins were greatly altered in the *mn*1. These results have not only improved our understanding of molecular mechanisms underlying the *mn*1 phenotype, but also laid a foundation for further studies on characterizing novel proteins. Future studies on many of these proteins will greatly enhance our knowledge on resource allocation and tolerance to biotic and abiotic stress in developing seeds.

SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00211/abstract

Figure S1 | Interactive graph for the analysis of enriched biological functions in (A) SPs and (B) CWAPs with REVIGO tool kit.

Table S1 | Gene accession numbers and the primers for genes investigated in the present study.

Table S2 | Total protein ID of soluble proteins.

Table S3 | Total protein ID of cell wall associated proteins.

Table S4 | agriGO results for soluble proteins.

Table S5 | agriGO results for cell wall associated proteins.

Table S6 | Shared differentially expressed proteins in SPs and CWAPs.

Table S7 | KEGG identifiers for secretory proteins from TargetP.

REFERENCES

Ali, N. A., McKay, M. J., and Molloy, M. P. (2010). Proteomics of Smad4 regulated transforming growth factor-beta signaling in colon cancer cells. *Mol Biosyst.* 6, 2332–2338. doi: 10.1039/c0mb00016g

Barratt, D. H., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., et al. (2009). Normal growth of Arabidopsis requires cytosolic invertase but not sucrose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13124–13129. doi: 10.1073/pnas.0900689106

Carlson, S. C., and Chourey, P. S. (1999). A re-evaluation of the relative roles of two invertases, INCW2 and IVR1, in developing maize kernels and other tissues. *Plant Physiol.* 121, 1025–1035. doi: 10.1104/pp.121.3.1025

Chen, M., Ying, W., Song, Y., Liu, X., Yang, B., Wu, S., et al. (2007). Analysis of human liver proteome using replicate shotgun strategy. *Proteomics* 7, 2479–2488. doi: 10.1002/pmic.200600338

Chen, S., and Harmon, A. C. (2006). Advances in plant proteomics. *Proteomics* 6, 5504–5516. doi: 10.1002/pmic.200600143

Cheng, W. H., and Chourey, P. S. (1999). Genetic evidence that invertase-mediated release of hexoses is critical for appropriate carbon partitioning and normal seed development in maize. *Theor. Appl. Genet.* 98, 485–495. doi: 10.1007/s001220051096

Cheng, W. H., Taliercio, E. W., and Chourey, P. S. (1996). The Miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell* 8, 971–983. doi: 10.1105/tpc.8.6.971

Chourey, P. S., Jain, M., Li, Q. B., and Carlson, S. J. (2006). Genetic control of cell wall invertase in developing endosperm of maize. *Planta* 223, 159–167. doi: 10.1007/s00425-005-0039-5

Chourey, P. S., Li, Q. B., and Kumar, D. (2010). Sugar–hormone cross-talk in seed development: two redundant pathways of IAA biosynthesis are regulated differentially in the invertase-deficient *miniature1* (*mn1*) seed mutant in maize. *Mol. Plant* 3, 1026–1036. doi: 10.1093/mp/ssq057

Chourey, P. S., Li, Q. B., and Cevallos-Cevallos, J. (2012). Pleiotropy and its dissection through a metabolic gene Miniature1 (*Mn1*) that encodes a cell wall

invertase in developing seeds of maize. *Plant Sci.* 184, 45–53. doi: 10.1016/j.plantsci.2011.12.011

Costa, L. M., Yuan, J., Rouster, J., Paul, W., Dickinson, H., and Gutierrez-Marcos, J. F. (2012). Maternal control of nutrient allocation in plant seeds by genomic imprinting. *Curr. Biol.* 22, 160–165. doi: 10.1016/j.cub.2011.11.059

Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* 38, 64–70. doi: 10.1093/nar/gkq310

Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994). Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem. Sci.* 19, 331–336. doi: 10.1016/0968-0004(94)90072-8

Gallardo, K., Firnhaber, C., Zuber, H., Héricher, D., Belghazi, M., Henry, C., et al. (2007). A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds: evidence for metabolic specialization of maternal and filial tissues. *Mol. Cell Proteomics* 6, 2165–2179. doi: 10.1074/mcp.M700171-MCP200

Gómez, E., Royo, J., Muñiz, L. M., Sellam, O., Paul, W., Gerentes, D., et al. (2009). The maize transcription factor myb-related protein-1 is a key regulator of the differentiation of transfer cells. *Plant Cell* 21, 2022–2035. doi: 10.1105/tpc.108.065409

Hajduch, M., Hearne, L. B., Miernyk, J. A., Casteel, J. E., Joshi, T., Agrawal, G. K., et al. (2010). Systems analysis of seed filling in Arabidopsis: using general linear modeling to assess concordance of transcript and protein expression. *Plant Physiol.* 152, 2078–2087. doi: 10.1104/pp.109.152413

Houston, N. L., Fan, C., Xiang, J. Q., Schulze, J. M., Jung, R., and Boston, R. S. (2005). Phylogenetic analyses identify 10 classes of the protein disulfide isomerase family in plants, including single-domain protein disulfide isomerase-related proteins. *Plant Physiol.* 137, 762–778. doi: 10.1104/pp.104.056507

Hurkman, W. J., and Tanaka, C. K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* 81, 802–806. doi: 10.1104/pp.81.3.802

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res* 40(Database issue), D109–D114. doi: 10.1093/nar/gkr988

Kang, B. H., Xiong, Y., Williams, D. S., Pozueta-Romero, D., and Chourey, P. S. (2009). Miniature1-encoded cell wall invertase is essential for assembly and function of wall-in-growth in the maize endosperm transfer cell. *Plant Physiol.* 151, 1366–1376. doi: 10.1104/pp.109.142331

Koh, J., Soltis, P. S., and Soltis, D. E. (2010). Homolog loss and expression changes in natural populations of the recently and repeatedly formed allotetraploid *Tragopogon mirus* (Asteraceae). *BMC Genomics* 11:97. doi: 10.1186/1471-2164-11-97

Koller, A., Washburn, M. P., Lange, B. M., Andon, N. L., Deciu, C., Haynes, P. A., et al. (2002). Proteomic survey of metabolic pathways in rice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11969–11974. doi: 10.1073/pnas.172183199

Lan, P., Li, W., Wen, T. N., Shiau, J. Y., Wu, Y. C., Lin, W., et al. (2011). iTRAQ protein profile analysis of Arabidopsis roots reveals new aspects critical for iron homeostasis. *Plant Physiol.* 155, 821–834. doi: 10.1104/pp.110.169508

LeClere, S., Schmelz, E. A., and Chourey, P. S. (2008). Cell wall invertase-deficient miniature 1 kernels have altered phytohormone levels. *Phytochemistry* 69, 692–699. doi: 10.1016/j.phytochem.2007.09.011

LeClere, S., Schmelz, E. A., and Chourey, P. S. (2010). Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. *Plant Physiol.* 153, 306–318. doi: 10.1104/pp.110.155226

Lee, J., and Koh, H. J. (2011). A label-free quantitative shotgun proteomics analysis of rice grain development. *Proteome Sci.* 9, 61. doi: 10.1186/1477-5956-9-61

Massonneau, A., Condamine, P., Wisniewski, J. P., Zivy, M., and Rogowsky, P. M. (2005). Maize cystatins respond to developmental cues, cold stress and drought. *Biochim. Biophys. Acta* 1729, 186–199. doi: 10.1016/j.bbaexp.2005.05.004

Méchin, V., Thévenot, C., Le Guilloux, M., Prioul, J. L., and Damerval, C. (2007). Developmental analysis of maize endosperm proteome suggests a pivotal role for pyruvate orthophosphate dikinase. *Plant Physiol.* 143, 1203–1219. doi: 10.1104/pp.106.092148

Miernyk, J. A., and Hajduch, M. (2011). Seed proteomics. *J. Proteomics.* 74, 389–400. doi: 10.1016/j.jprot.2010.12.004

Miller, M. E., and Chourey, P. S. (1992). The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. *Plant Cell* 4, 297–305. doi: 10.1105/tpc.4.3.297

Neilson, K. A., Mariano, M., and Haynes, P. A. (2011). Quantitative proteomic analysis of cold-responsive proteins in rice. *Proteomics* 11, 1696–1706. doi: 10.1002/pmic.201000727

Nelson, D. L., and Cox, M. M. (2008). *Lehninger Principles of Biochemistry,* 5th edition. New York, NY: W. H. Freeman and Co.

Owiti, J., Grossmann, J., Gehrig, P., Dessimoz, C., Laloi, C., Hansen, M. B., et al. (2011). iTRAQ-based analysis of changes in the cassava root proteome reveals pathways associated with post-harvest physiological deterioration. *Plant J.* 67, 145–156. doi: 10.1111/j.1365-313X.2011.04582.x

Pugh, D. A., Offler, C. E., Talbot, M. J., and Ruan, Y.-L. (2010). Evidence for the role of transfer cells in the evolutionary increase in seed and fiber biomass yield in cotton. *Mol. Plant* 3, 1075–1086. doi: 10.1093/mp/ssq054

Ralser, M., Wamelink, M. M., Kowald, A., Gerisch, B., Heeren, G., Struys, E. A., et al. (2007). Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *J. Biol.* 6, 10. doi: 10.1186/jbiol61

Ruan, Y-L, Llewellyn, D. J., and Furbank, R. T. (2003). Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation and seed development. *Plant Cell* 15, 952–964. doi: 10.1105/tpc.010108

Scanlon, M. J., and Takacs, E. M. (2009). "Kernel biology," in *Handbook of Maize, Its Biology*, eds J. Bennetzen and S. Hake (New York, NY: Springer), 121–129.

Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS ONE* 6:e21800. doi: 10.1371/journal.pone.0021800

Taliercio, E. W., Kim, J. Y., Mahe, A., Shanker, S., Choi, J., Cheng, W. S., et al. (1999). Isolation, characterization and expression analyses of two cell wall invertase genes in maize. *J. Plant Physiol.* 155, 197–204. doi: 10.1016/S0176-1617(99)80007-8

Van Aken, O., Whelan, J., and Van Breusegem, F. (2010). Prohibitins: mitochondrial partners in development and stress response. *Trends Plant. Sci.* 15, 275–282. doi: 10.1016/j.tplants.2010.02.002

van der Linde, K., Hemetsberger, C., Kastner, C., Kaschani, F., Van der Hoorn, R. A. L., Kumlehn, J, et al. (2012). A maize cystatin suppresses host immunity by inhibiting apoplastic cysteine proteases. *Plant Cell* 24, 1285–1300. doi: 10.1105/tpc.111.093732

Vilhar, B., Kladnik, A., Blejec, A., Chourey, P. S., and Dermastia, M. (2002). Cytometrical evidence that the loss of seed weight in the miniature1 seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. *Plant Physiol.* 129, 23–30. doi: 10.1104/pp.001826

Xiong, Y., Li, Q. B., Kang, B. H., and Chourey, P. S. (2011). Discovery of genes expressed in basal endosperm transfer cells in maize using 454 transcriptome sequencing. *Plant Mol. Biol. Rep.* 29, 835–847. doi: 10.1007/s11105-011-0291-8

Zhu, M., Simons, B., Zhu, N., Oppenheimer, D. G., and Chen, S. (2010). Analysis of abscisic acid responsive proteins in *Brassica napus* guard cells by multiplexed isobaric tagging. *J. Proteomics* 73, 790–805. doi: 10.1016/j.jprot.2009.11.002

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Does Don Fisher's high-pressure manifold model account for phloem transport and resource partitioning?

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The pressure flow model of phloem transport envisaged by Münch (1930) has gained wide acceptance. Recently, however, the model has been questioned on structural and physiological grounds. For instance, sub-structures of sieve elements may reduce their hydraulic conductances to levels that impede flow rates of phloem sap and observed magnitudes of pressure gradients to drive flow along sieve tubes could be inadequate in tall trees. A variant of the Münch pressure flow model, the high-pressure manifold model of phloem transport introduced by Donald Fisher may serve to reconcile at least some of these questions. To this end, key predicted features of the high-pressure manifold model of phloem transport are evaluated against current knowledge of the physiology of phloem transport. These features include: (1) An absence of significant gradients in axial hydrostatic pressure in sieve elements from collection to release phloem accompanied by transport properties of sieve elements that underpin this outcome; (2) Symplasmic pathways of phloem unloading into sink organs impose a major constraint over bulk flow rates of resources translocated through the source-path-sink system; (3) Hydraulic conductances of plasmodesmata, linking sieve elements with surrounding phloem parenchyma cells, are sufficient to support and also regulate bulk flow rates exiting from sieve elements of release phloem. The review identifies strong circumstantial evidence that resource transport through the source-path-sink system is consistent with the high-pressure manifold model of phloem transport. The analysis then moves to exploring mechanisms that may link demand for resources, by cells of meristematic and expansion/storage sinks, with plasmodesmal conductances of release phloem. The review concludes with a brief discussion of how these mechanisms may offer novel opportunities to enhance crop biomass yields.

Keywords: Bulk flow, hydraulic conductance, hydrostatic pressure, plasmodesmata, phloem transport, symplasmic phloem unloading, resource partitioning

INTRODUCTION

The pressure flow model of phloem transport (Münch, 1930) has gained wide acceptance. The model envisages that an osmotically-generated differential in hydrostatic pressure propels a bulk flow of phloem sap through the sieve tube (ST) system linking photosynthetic sources leaves with heterotrophic sink organs. Within sieve elements (SEs) of source leaf collection phloem, sugars (sucrose, polyols or raffinose family oligosaccharides) accumulate to high concentrations (up to 1 M). This drives an osmotic uptake of water to generate relatively high hydrostatic pressures within the SEs. Conversely unloading of osmotica from SEs, located in release phloem of sinks, causes an osmotic withdrawal of water and a consequent lowering of their hydrostatic pressures. Thus, the Münch model predicts that bulk flow of phloem sap through STs is driven by differences in hydrostatic pressures located within source and sink SEs.

Experimental observations provide persuasive support for phloem sap moving through STs by bulk flow (e.g., van Bel and Hafke, 2005) driven by hydrostatic pressure heads generated by phloem loading in source leaves (e.g., Gould et al., 2005). Recently, however, aspects of the Münch pressure flow model have been questioned on structural and physiological grounds. For instance, the Hagen-Poiseuille Law (see Equation 1), describing solvent flow driven through a pipe by a hydrostatic pressure difference (ΔP) for a given viscosity (η), predicts that variation in flow path geometries of STs (length–L and, in particular, radius–r), will alter their hydraulic conductances (L_o and see Equation 2) and hence impact axial volume flow rates (R_v) through them.

$$R_v = \pi r^4 \Delta P / 8\eta L \quad (1)$$

and where

$$L_o = \pi r^4 / 8\eta L \quad (2)$$

The observed absence of any impact on axial volume flux (i.e., axial velocity derived from R_v expressed on the internal cross-sectional area through which solvent flow is occurring) by variations in sieve pore radii (Mullendore et al., 2010) or extent of P-protein occlusion of SE lumens (Froelich et al., 2011) has led to the suggestion that yet-to-be identified phloem transport properties are being overlooked by the Münch model (Mullendore et al., 2010; Froelich et al., 2011). A similar conclusion has been

drawn from the observation that magnitudes of hydrostatic pressure gradients within STs of tall trees are inadequate to account for observed rates of bulk flow through these pipelines (Turgeon, 2010 but c.f. Jensen et al., 2012).

A variant of the Münch pressure flow model, the high-pressure manifold model of phloem transport (Fisher, 2000 and see **Figure 1**), may serve to reconcile at least some of these questions. To this end, the high-pressure manifold model of phloem transport is evaluated in relation to known properties and characteristics of phloem transport. The review commences by distilling out the key features of the high-pressure model of phloem transport followed by evaluating each of these against current knowledge of phloem transport biology.

HIGH-PRESSURE MANIFOLD MODEL OF PHLOEM TRANSPORT

Key elements of the high-pressure manifold model of phloem transport articulated by Fisher (2000) and captured in **Figure 1** are:

1. High hydrostatic pressures are maintained within STs throughout the length of the transport phloem with minimal decreases between collection and release phloem SEs (**Figure 1**). Magnitudes of these pressures are primarily generated by activities of processes determining the level to which solutes are accumulated in collection phloem SEs.
2. Large differentials in hydrostatic pressure between SEs and phloem parenchyma cells of release phloem are generated by phloem sap being expelled, by bulk flow, through paths of low hydraulic conductance (i.e., the manifold and see Equation 2) provided by plasmodesmata interconnecting these cells.
3. Phloem unloading rates are regulated by hydraulic conductances (Equation 2) of the unloading pathway rather than the axial hydrostatic pressure differential (Equation 1). In this context, the latter is comparable between a source and an array of sinks irrespective of their rates of phloem unloading (**Figure 1**).
4. A considerable component of the regulation of resource partitioning between sinks is mediated by the relative hydraulic conductances (Equation 2) of symplasmic unloading pathways linking SEs with phloem parenchyma cells in the release phloem (**Figure 1**).

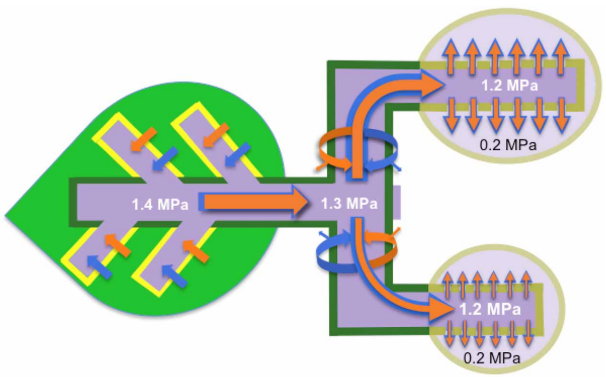

FIGURE 1 | High-pressure manifold model that describes phloem transport from source to sink and partitioning of resources (water and dissolved solutes) between sinks. Sucrose is loaded (brown arrows) into the collection phloem (minor veins; yellow borders) of source leaves to high concentrations (dark purple) that drives an osmotic uptake of water (blue arrows). Walls of the collection phloem SEs resist the volume change with a consequent development of high hydrostatic pressures (example given, 1.4 MPa and see **Table 1**). STs form conduits interconnecting sources (dark green) to sinks (light purple) in a supercellular symplasm comprising collection, transport (dark green) and release (khaki) phloem. Hydrostatic pressures, generated in collection phloem SEs, are rapidly transmitted throughout the entire ST system and maintained by pressure-dependent retrieval of leaked sucrose and hence water (curved brown and blue arrows respectively). Thus STs are conceived to function as high-pressure conduits rendering resources equally available throughout a plant. Transported resources are unloaded from the release phloem SE/CC complexes as a bulk flow through high resistance (low conductance L_o and see Equation 2) plasmodesmal pathways into the sink cells. SE/CC unloading imposes the greatest constraint over resource flow through the source-transport-sink pathway. As a consequence, resource partitioning between sinks is finely regulated by their relative hydraulic conductances of plasmodesmata linking SE/CC complexes with the surrounding phloem parenchyma cells.

Our analysis of the high-pressure manifold model of phloem transport primarily rests on evaluating the extent to which the model is consistent with known properties of resource flow through the phloem. Of necessity much of this analysis will focus on phloem transport in herbaceous plants where most experimental studies have been undertaken. However, with appropriate caveats, the analysis is extended to phloem transport in trees.

HYDROSTATIC PRESSURE GRADIENTS IN SIEVE TUBES—STEEP OR MODERATE?

Bulk flow in the Münch model is conceived to be restricted to STs alone in which substantial hydrostatic pressure gradients develop from their source to sink ends. In contrast, the high-pressure manifold model (Fisher 2000 and see **Figure 1**) envisages the entire plant ST system is strongly pressurized with a minimal differential in hydrostatic pressures between collection and release phloem SEs. However, a major hydrostatic pressure differential occurs as a result of the substantial frictional drag imposed on an ongoing bulk flow through plasmodesmata (the manifold) interconnecting release phloem SEs with adjacent parenchyma

Table 1 | Measures of sieve tube hydrostatic pressures (MPa) using micromanometers glued to severed aphid stylets inserted into translocating sieve tubes, in specified plant organs and species.

Plant species	Organ	Hydrostatic pressure	References
Hordeum vulgare (Barley)	Leaf	0.8–1.4	Gould et al., 2005
	Root	1.62 ± 0.05	Gould et al., 2004a
Sonchus oleraceus (Sow thistle)	Leaf	1.0–1.5	Gould et al., 2005
	Stem	0.7–1.0	Gould et al., 2004b
Triticum aestivum (Wheat)	Peduncle	2.35 ± 0.56	Fisher and Cash-Clark, 2000b
	Grain	1.16 ± 0.26	
Salix babylonica (Willow)	Leaf	0.51–0.93	Wright and Fisher, 1980
	Bark strip	0.47–1.2	Wright and Fisher, 1983

cells (**Figure 1**). Literature reporting measures of ST pressures to test the proposition that hydrostatic pressure gradients between source and sink ends of ST systems are moderate is reviewed below.

Early attempts to directly measure hydrostatic pressures of STs [e.g., by manometry or pressure transducers using the phloem pressure probe described by Hammel (1968)] or indirectly (as the difference between ST sap water and solute potentials) were considered to be compromised respectively by technical shortcomings (e.g., an incomplete seal around the inserted Hammel hypodermic syringe needle, and its destruction of STs) or the assumption that STs were at water equilibrium with their surrounding apoplasms (Milburn and Kallarackal, 1989). Nevertheless, estimates of ST hydrostatic pressures, deduced from measures of ST sap osmotic potentials and xylem water potentials, closely approximate those obtained using manometry or pressure transducers (e.g., see Wright and Fisher, 1980; Sovonick-Dunford et al., 1981). These findings indicate that, under most circumstances, STs are at water potential equilibrium (see Thompson and Holbrook, 2003).

The most reliable direct measures of SE hydrostatic pressures have been obtained using a methodology, developed by Wright and Fisher (1980), consisting of micromanometers glued to severed aphid stylets inserted into conducting STs. The challenging nature of the technique accounts for the few published studies, mostly carried out using herbaceous species. Averaged hydrostatic pressure values for leaves and stems need to be interpreted with caution, as it is likely that ST hydrostatic pressures oscillate with fluctuations in xylem water potentials caused by changes in transpiration loads (Lee, 1981). Such temporal oscillations, at least in part, account for the spread of recorded hydrostatic pressures in STs (**Table 1**). Unfortunately, to our knowledge, there are not any reported systematic examinations of ST hydrostatic pressures along source to sink axial pathways using aphid micromanometry. However, in broad terms, published data (**Table 1**) suggest that, if a source to sink gradient in hydrostatic pressure exists within STs, the gradient is minimal for herbaceous plants (and also see Turgeon, 2010). This feature, coupled with high ST hydrostatic pressures of 1.0 MPa, supports the contention that phloem transport in herbaceous species is consistent with the high-pressure manifold model (Fisher, 2000 and see **Figure 1**). The puzzling exception to this generalization is the very large and negative differential between SE hydrostatic pressures located in peduncles and the crease vein of wheat grains (**Table 1**) for which there is no plausible explanation at this time (see Fisher and Cash-Clark, 2000b).

Whether ST hydrostatic pressures and their axial gradients in trees comply with the high-pressure manifold model of phloem transport is rendered problematic by the paucity of suitable data sets. The only available measures of hydrostatic pressures determined by aphid stylet micromanometry are for willow (Wright and Fisher, 1980, 1983) and these are 0.2–0.5 MPa lower than those reported for herbaceous species (**Table 1**). However, their lower hydrostatic pressures may arise from the experimental conditions under which these were measured (Turgeon, 2010). For willow, leaf measures of ST hydrostatic pressures were determined using potted plants equilibrated under laboratory light well below the light compensation point (Wright and Fisher, 1980) and stem measures were carried out on isolated bark strips (Wright and Fisher, 1983). Both these experimental conditions would be expected to decrease the pool size of sugars available for phloem loading causing phloem sap osmotic potentials to be elevated thus lowering ST hydrostatic pressures. An assertion supported by measures of ST hydrostatic pressures ranging from 0.5 to 1.7 MPa in stems of 15 m tall white ash (*Fraxinus americana*) trees during the photoperiod (Lee, 1981; Sovonick-Dunford et al., 1981). These values are probably underestimates of their actual ST hydrostatic pressures since they were obtained using the Hammel (1968) pressure recording system (Milburn and Kallarackal, 1989). Furthermore phloem sap osmotic potentials of willow and poplar were much lower than those found in herbaceous species raised under high light conditions (Turgeon, 2010). Thus tree ST hydrostatic pressures are considered to be comparable to, or even greater than, those of herbaceous species.

Measures of ST hydrostatic pressure of willow leaf and stem by aphid stylet micromanometry (**Table 1**), and of white ash stems at different heights by the Hammel (1968) device (Lee, 1981), do not indicate the presence of any substantial axial gradients in ST hydrostatic pressure. However, source to sink gradients in phloem sap sugar concentrations have been detected in young (Pate and Arthur, 2000) but not well-established (Merchant et al., 2012) *Eucalyptus globulus* trees growing in plantations. Overall, the available data suggest that, at least in certain circumstances, phloem transport in trees can exhibit high ST hydrostatic pressures with minimal gradients between source and sink ends of the ST system consistent with the high-pressure manifold model (**Figure 1**).

Recent modeling of Münch pressure flow to optimize translocation velocity led to deriving a simple scaling relationship between ST radii with leaf (loading) and stem (transport) lengths that accounts for phloem translocation by Münch pressure flow in plant heights spanning several orders of magnitude (Jensen et al., 2011, 2012). In addition, earlier modeling predicted minimal hydrostatic pressure differentials between source and sink were essential for integrated functioning of the phloem transport system throughout the plant body (Thompson, 2006 and references cited therein). The latter model mimics the high-pressure model of phloem translocation and, of necessity, relies on ST conductances (Equation 2) scaling with plant height (see Jensen et al., 2012). However, as argued below, other features of phloem transport also may contribute to minimizing the hydrostatic pressure differential between source and sink ends of the phloem pathway.

CONDITIONS FOR HIGH HYDROSTATIC PRESSURES THROUGHOUT SIEVE TUBE SYSTEM WITH MINIMAL GRADIENTS FROM SOURCE TO SINK

In addition to highly conductive STs, high hydrostatic pressures throughout the ST system accompanied by minimal hydrostatic pressure gradients from source to sink depend upon:

POTENTIAL CAPACITY OF PHLOEM LOADING EXCEEDS PHLOEM UNLOADING

Under conditions of source limitation, where phloem-unloading rates of sugars (and other phloem sap solutes) exceed the potential

capacity of phloem loading, phloem sap concentrations of sugars, accompanied by ST hydrostatic pressures, decline until a new equilibrium between loading and unloading rates are reached. This condition could cause a pronounced axial gradient in ST hydrostatic pressure from source to sink to develop. The corollary is sink limitation whereby the potential capacity for phloem loading of sugars exceeds that of phloem unloading. For this condition, any increase in rates of phloem unloading is met by a commensurate increase in phloem loading. This condition would sustain high phloem sap sugar concentrations and hence ST hydrostatic pressures throughout the system.

There are no direct measures of rates of phloem loading and unloading. However, there is considerable indirect evidence suggesting that potential capacities of leaf photosynthesis arranged in series with phloem loading exceed those of phloem unloading into expanding and storage sinks. For instance, under optimal environmental conditions for leaf photosynthesis, increasing sink/source ratios by experimentally attenuating sugar export from a portion of leaves (by their removal or shading) results in elevated rates of sugar export from the untreated leaves of trees (Pinkard et al., 2011), monocots (McCormick et al., 2006) and herbaceous eudicots (Borrás et al., 2004). Similarly transgenic-mediated increases in sink capacities to store or utilize sugars, also lead to enhancing leaf photosynthesis and phloem loading (e.g., Smidansky et al., 2007; Wu and Birch, 2007). Thus under optimal conditions, plant growth tends to be sink-limited and hence phloem transport likely functions as a high pressure manifold system (see above). However, source-limitation can arise in certain circumstances including an excess of reproductive development (Qian et al., 2012), through abiotic stress (Körner, 2003) or during periods of replenishing stores of non-structural carbohydrates depleted by episodes of abiotic stress in perennial woody plants (Sala et al., 2012). Under conditions of source limitation, phloem hydrostatic pressures are predicted to be depressed and exhibit a more pronounced axial source to sink gradients in ST hydrostatic pressures. Thus, the influence of a high-pressure manifold system would be diminished under source limitation and hydraulic conductances (Equation 2) of STs could assume an increasing influence.

AXIAL HYDRAULIC CONDUCTANCES OF SIEVE TUBES ARE CONSIDERABLY HIGHER THAN THOSE OF PHLOEM UNLOADING PATHWAYS

If exit of phloem sap from SEs at sinks dominates regulation of axial flow through STs from source to sink, it follows that any axial gradient in ST hydrostatic pressure would be minimized consistent with the high-pressure manifold model of phloem transport (**Figure 1** and see Point 1). That ST structure, and particularly sieve plates, do not exert much influence over axial transport through them has been demonstrated by very elegant structural studies to obtain estimates of ST hydraulic conductivities [hydraulic conductance (see Equation 2) expressed on cross-sectional area basis through which bulk flow occurs] for a variety of plant species with differing sieve pore dimensions (Mullendore et al., 2010). The Hagen-Poiseuille Law (see Equation 1) predicts that, if ST conductivities were regulating axial transport, phloem transport velocities (volume flux—R_v of Equation (1) expressed on a cross-sectional area basis i.e., πr^2) would vary directly with sieve pore radius raised to the second power. However, across a cohort of test species, estimates of phloem transport velocities were found to exhibit an inverse rather than the predicted direct relationship with their estimated ST hydraulic conductivities (Mullendore et al., 2010). This finding suggests that control of axial flow of phloem sap through STs is located elsewhere in the system. Similar conclusions were reached using an Arabidopsis T-DNA knock-out *seor1* mutant which had a reduced P-protein network (SEOR1 proteins) in STs (Froelich et al., 2011) Consistent with an absence of control being exercised by ST hydraulic conductances, phloem transport velocities in wild type and *seor1* mutant plants were found to be identical (Froelich et al., 2011).

More direct evidence that STs have spare transport capacity to support axial flow comes from physiological studies in which partial (up to ca 50%) surgical removal of phloem cross-sectional area in stems was found to exert little influence over axial flow rates in both woody plants (e.g., Mason and Maskell, 1928) and monocots (e.g., Wardlaw and Moncur, 1976). In contrast, large, and positive responses of axial phloem transport rates to surgical excision of release phloem are consistent with a major control over axial transport through the ST system being exercised by phloem unloading. A striking example of this phenomenon takes advantage of castor oil (*Ricinus communis*) plants whose STs do not seal upon severance, allowing phloem transport to continue. Hence rates of phloem transport can be estimated by measuring quantities (volume; mass) of phloem sap exuded from cut surfaces across specified times. The estimated transport rates can be expressed as ST fluxes based on measures of their cross-sectional areas through which axial transport takes place (Milburn and Kallarackal, 1989). In this case, excision of the apical fruit from a raceme of developing *Ricinus* fruits caused ST fluxes of sucrose through the pedicel stump to increase by 19-fold compared to pedicel ST fluxes when the fruit was attached (Kallarackal and Milburn, 1984). Fruit excision would remove any impedance imposed by phloem unloading on axial flow through STs and increase the hydrostatic pressure differential driving axial flow to the cut pedicel surface. Impedance conferred by phloem unloading cannot be estimated directly but changes in the hydrostatic pressure differential driving axial flow through pedicel STs can be estimated as follows. ST hydrostatic pressures of *Ricinus*, determined from osmotic and water potentials, approximate 1.0 MPa (Smith and Milburn, 1980a). Fruit excision would cause hydrostatic pressures of severed SEs located at surfaces of cut pedicels to drop to zero (i.e., atmospheric pressure). Lets assume that attached *Ricinus* fruits conform with the high-pressure manifold model of phloem transport whereby bulk flow continues symplasmically from release phloem SEs into adjacent sink cells with turgors in the range of 0.1–0.2 MPa (Saladié et al., 2007; Wada et al., 2009). Under these conditions, hydrostatic pressure differential driving bulk flow through the STs and ultimately through plamodesmata into fruit sink cells would be 0.9 or 0.8 MPa respectively. Thus, on reducing the sink-end hydrostatic pressure to zero, upon pedicel severance, the hydrostatic pressure differential driving axial flow through the pedicel STs would increase by 10 or

20% respectively (see Equation 1). As a consequence, the remaining 17.8- to 14.2-fold increase in the ST flux of sucrose elicited by fruit excision (Kallarackal and Milburn, 1984) must result from removing a considerable resistance to bulk flow imposed by SE unloading through a symplasmic pathway. This outcome is consistent with the high-pressure manifold model of phloem transport (**Figure 1**). Similar magnitudes of phloem transport rate increases were observed in wheat inflorescences when developing grains were detached and rates of phloem sap exuded from the broken pedicel surfaces determined (Fisher and Gifford, 1986).

An exemption to exit from release phloem dominating overall flow from source to sink is the influence imposed by hydraulic conductances of differentiating phloem paths linked with meristematic sinks. Root tips provide an opportunity to gain insights into the influence of the axial hydraulic conductances of differentiating symplasmic phloem paths (Stadler et al., 2005a). For example, as the *Zea mays* root meristem is approached, osmotic potentials of SE sap decline precipitously from axial steady levels of 4 MPa to 1.3–2.1 MPa (Warmbrodt, 1987). This decline suggests a marked reduction in hydraulic conductance (see Equation 2) of the axial phloem path that is linked with an increasing portion of differentiating SEs and provascular elements. Moreover, the decrease in osmotic potential along the axial path was ca 2-fold greater than the decrease in osmotic pressure from protophloem SEs to reach meristematic cells (Warmbrodt, 1987). This suggests a greater influence by the differentiating axial pathway on delivery to apical meristems rather than subsequent movement through the phloem-unloading route. These observations are consistent with phloem imported GFP-fusion proteins (ranging in molecular size from 36 kDa to 67 kDa) being restricted to protophloem SEs and not being able to enter differentiating protophloem SEs of Arabidopsis root tips (Stadler et al., 2005a).

TURGOR HOMEOSTASIS OF THE SIEVE TUBE SYSTEM

A prerequisite for the high-pressure manifold model of phloem transport (**Figure 1**) is for STs, extending from collection phloem in leaves through the transport phloem to release phloem in sinks, to function as cohesive units that sustain high hydrostatic pressures throughout their lengths with minimal source to sink gradients. This prerequisite depends upon satisfying the following conditions:

A rapid signaling system to convey local changes in solute demand throughout the entire sieve tube system

Increases in sink/source ratios, by blocking photosynthesis in all but one leaf (Fondy and Geiger, 1980), releasing phloem content through stem incisions (Smith and Milburn, 1980a) or selective warming of sink organs (Minchin et al., 2002), can cause rapid (instantaneous to within minutes) increases in rates of phloem export of recently-fixed photoassimilates from source leaves. The rapidity at which rates of phloem export from source leaves respond to altered sink/source ratios is considered to depend upon the degree of buffering of ST content by exchange from solute pools located along the transport phloem (Minchin et al., 2002 and see **Figure 1**). Indeed, buffering transport phloem content from these solute pools can meet altered sink demand without any need for change in export rates of recently-fixed photoassimilates (e.g., Fondy and Geiger, 1980). The effect is at its most extreme for the stem incision model whereby hydrostatic pressures in STs, upon severance, instantaneously fall to zero occasioning a decrease in their water potentials to drive an influx of water that would otherwise dilute their phloem sap concentrations (Smith and Milburn, 1980a). The change in sap hydrostatic pressure-concentration is rapidly propagated as a wave (Thompson, 2006) along the ST conduit to stimulate phloem loading (Smith and Milburn, 1980a). In this context, once phloem transport reaches a new steady-state in response to enhanced sink demand, osmotic concentrations of phloem sap remain unaltered and increased transport rates were accounted for by commensurate changes in transport velocity (Wardlaw and Moncur, 1976; Smith and Milburn, 1980a). Consistent with theory predicting the rapidity in axial propagation of pressure-concentration waves in response to localized changes in solute loading/unloading (Thompson, 2006; Mencuccini and Hölttä, 2010), remote changes in phloem transport rates, following localized heat ringing of sunflower stems, were conferred by a signal travelling at an axial velocity an order of magnitude greater than that of phloem transport (Watson, 1976 and also see Münch, 1930). Transmission of pressure-concentration waves depends upon elasticity and hydraulic conductances of STs (Thompson, 2006 and references cited therein). Whether ST conductances are capable of supporting rapid propagation of pressure-concentration waves over long-distances in tall trees is uncertain (Thompson, 2006), but considered likely (Mencuccini and Hölttä, 2010).

A mechanism for homeostasis of sieve tube hydrostatic pressures and osmotic solute concentrations

That pressure-concentration waves convey information to the entire ST system depends on high ST hydrostatic pressures and osmotic solute concentrations (Thompson and Holbrook, 2003) and hence concentrations of each major osmotic species, sucrose and potassium. In this context, apoplasmic phloem loading in leaves is negatively regulated by ST hydrostatic pressures in *Ricinus* (Smith and Milburn, 1980b), *Phaseolus coccinius* (Daie and Wyse, 1985), *Pisum sativum* (Estruch et al., 1989) and by an apoplasmic sucrose concentration specific mode in leaves of the halophyte, *Beta vulgaris* (Chiou and Bush, 1998). Passive loading in tall trees offsets decreasing water potentials with plant height through osmoregulatory activities of their mesophyll cells (Fu et al., 2011). To our knowledge, regulation of loading by the polymer trap mechanism in response to sink demand has not been investigated. However, at least some species, loading by the polymer trap mechanism, exhibit mixed loading and contain an element of apoplasmic loading of sucrose. Included amongst these species are herbaceous eudicots (Voitsekhovskaja et al., 2009; Gil et al., 2011). The ability of these polymer trap species to load sucrose apoplasmically provides opportunities for ST osmoregulation. Such a strategy may account for the positive relationship between decreasing leaf water potentials and considerable osmotic adjustment of phloem sap by elevated sucrose concentrations in trees of raffinose

translocating *Eucalyptus globulus* growing across a rainfall gradient (Merchant et al., 2010). An inverse relationship between plasma membrane H^+-ATPase activity localized to SE-CC complexes and ST hydrostatic pressure (Estruch et al., 1989) provides a unifying model for turgor homeostasis of STs. Thus, turgor-regulated shifts in H^+-ATPase activity generate adjustments in the proton motive force driving proton-coupled symport of solutes into SE-CC complexes and membrane potential driving uptake of cations through membrane channels and, in particular, potassium.

The phenomenon of turgor homeostasis extends to SE-CCs of the transport phloem. This is graphically illustrated by positioning a cold block on a test stem to transiently slow axial phloem transport and recording temporal changes in ST hydrostatic pressure by aphid stylectomy/pressure transducers located up- and down-stream of the cold block. Applying this approach to sow thistle, Gould et al. (2004b) found that ST hydrostatic pressures upstream of the temperature block transiently rose (by >0.7 MPa) on stem chilling before declining, within 2 min, to values slightly above the pre-chill levels prior to the recovery of axial flow (8 min). Re-warming stems occasioned hydrostatic pressures to rapidly return to pre-chill levels. Down-stream of the cold block, ST hydrostatic pressures rapidly fell on chilling by 0.25–0.5 MPa and thereafter remained steady or slowly recovered. Irrespective, on rewarming stems, ST hydrostatic pressures rapidly returned to pre-chilled levels at sites up- and down-stream of the cold block. These responses are consistent with a rapid turgor homeostasis mediated throughout the ST system. During these hydrostatic pressure transients, sap osmotic potentials and sucrose concentrations were unaltered. These responses indicate that turgor homeostasis relied on co-ordinated radial exchange of solutes and water between STs and their surrounding apoplasm (Thorpe et al., 2005 and see **Figure 1**). Membrane carriers and channels facilitate solute influx down proton motive forces or membrane potentials respectively (Tegeder et al., 2012). The resulting transients in water potential differences between SEs and their surrounding apoplasms drive radial water fluxes into, and from, transport phloem SEs through aquaporins to maintain ST turgor. This phenomenon also would ensure compensation for loss in ST hydrostatic pressure due to frictional drag of the ST walls as predicted by Hagen-Poiseuille Law (see Equation 1).

SYMPLASMIC PHLOEM UNLOADING BY BULK FLOW THROUGH A PLASMODESMAL MANIFOLD

The phenomenon of phloem unloading is not restricted to release phloem of terminal sinks such as apical meristems and developing reproductive (flowers, fruit, seeds) or storage (tubers, bulbs) organs. In particular, under high source/sink ratios, net resource flows occur from transport phloem of petioles, stems and roots into storage pools of their ground tissues in which transport phloem is embedded. A striking example of this phenomenon is accumulation of sucrose in storage parenchyma cells of sugarcane stems to concentrations that match those of the phloem sap (Patrick, 2012). Thus, consideration also will be given to those circumstances in which net resource unloading takes place from the transport phloem.

Phloem unloading may be arbitrarily divided into SE unloading and post-SE unloading which extends from phloem parenchyma cells to cellular sites of resource utilization or storage in non-vascular cells. For the purposes of evaluating the high-pressure manifold model of phloem transport, the primary focus is on SE unloading wherein resources reach the phloem parenchyma cells. However, some consideration, where relevant, will be given to post-SE unloading.

A central tenant of the high-pressure manifold model is that hydraulic conductances of the symplasmic unloading pathways, from SEs of release phloem, impose a major constraint over bulk flow of resources through the source-path-sink system (**Figure 1**). This distils to three key elements. First phloem sap is unloaded from SEs of release phloem through interconnecting plasmodesmata into surrounding phloem parenchyma cells and perhaps beyond. Second, phloem sap movement occurs as a bulk flow through plasmodesmata interconnecting SEs with surrounding phloem parenchyma cells. Third, hydraulic conductances of symplasmic unloading routes are significantly less than those supporting axial flow through STs from source to sink (and see Section Axial Hydraulic Conductances of Sieve Tubes are Considerably Higher than Those of Phloem Unloading Pathways).

PHLOEM UNLOADING IN MOST SINKS OCCURS THROUGH SYMPLASMIC ROUTES

The most definitive experimental approach to mapping phloem-unloading pathways has been through the use of membrane-impermeant fluorochromes introduced into phloem sap as membrane-permeant esters or as tags linked to transgenically-expressed foreign molecules under the control of companion cell specific promoters.

Root and shoot apices

Axial delivery pathways into apical meristems comprise protophloem SEs giving way to strands of provascular cells more proximal to the apical dome. Distribution of membrane-impermeant fluorochromes, imported through the phloem, have identified putative symplasmic unloading pathways extending from terminal ends of protophloem SEs through provascular stands to reach meristematic cells in root apices of monocots (e.g., Hukin et al., 2002) and eudicots (e.g., Stadler et al., 2005a). Further back from root tips, in root elongation zones, the symplasmic domain for phloem unloading is constrained to protophloem SE-CC complexes and vascular parenchyma cells (Hukin et al., 2002; Stadler et al., 2005a). Symplasmic phloem unloading also applies to shoot apical meristems, as demonstrated by membrane-impermeant fluorochromes moving radially from protophloem SEs in sink leaves of monocots (Haupt et al., 2001) and eudicots (Stadler et al., 2005a).

Phloem unloading in developing seeds—sieve element unloading is symplasmic but non-sieve element routes include an obligatory apoplasmic step

Vasculatures terminate at funicular/coat boundaries of small seeds (e.g., Arabidopsis—Stadler et al., 2005b) or permeate coats of larger seeds (e.g., cereals and grain legumes—Zhang et al.,

2007a). SE unloading of resources and their subsequent movement to specialized efflux cells follows symplasmic routes (Stadler et al., 2005b; Zhang et al., 2007a). Absence of symplasmic continuity between seed coat and filial (endosperm and embryo) tissues of developing seeds is bridged by specialized transport cells, located at maternal/filial interfaces, for resource efflux into, and influx from, seed apoplasmic spaces (Zhang et al., 2007a).

Tubers and fleshy fruits

Phloem unloading in developing potato tubers switches from an apoplasmic route in stolons to a symplasmic pathway in tubers (Viola et al., 2001). Phloem unloading pathways in certain fleshy fruits exhibit a reverse shift in phloem unloading pathway from symplasmic during their pre-storage phase to an apoplasmic one at the onset of sugar storage. Examples of this strategy include fruits of tomato (Patrick and Offler, 1996), grape (Zhang et al., 2006) and Chinese jujube –(Nie et al., 2010). In contrast, for other fleshy fruit an apoplasmic step in the phloem-unloading pathway is present throughout their development as illustrated for walnut (Wu et al., 2004), apple (Zhang et al., 2004) and cucumber (Hu et al., 2011). In all cases, phloem cell types in which phloem-imported fluorochromes are retained cannot be identified with confidence; a conclusion extending to Arabidopsis ovules (Werner et al., 2011). Based on plasma membrane surface areas and rates of unloading, phloem parenchyma cells are best placed to accommodate observed sucrose fluxes in developing tomato fruit (Offler and Horder, 1992).

Transport phloem

Sucrose continually escapes across plasma membranes of metaphloem SE-CC complexes by simple diffusion driven by large concentration differences between their lumens and surrounding apoplasm (Patrick, 1990 and see **Figure 1**). Furthermore, low plasmodesmal connectivity with adjoining phloem parenchyma cells, absence of membrane-impermeant dye movement and differing membrane potentials between the two cell types indicates metaphloem SE-CC complexes can be isolated symplasmically (e.g., Hayes et al., 1985; van Bel and van Rijen, 1994; Kempers et al., 1998). Apoplasmic unloading applies under low source/sink ratios (Patrick and Offler, 1996) where solute retrieval from the phloem apoplasm is dominated by SE-CC complexes over any other cell type by more than five-fold (Patrick and Turvey, 1981; Bieleski, 1966a,b; Deeken et al., 2002). Under high source/sink ratios, unloading from the transport phloem may switch to a symplasmic route (Grignon et al., 1989; Patrick and Offler, 1996; Stadler et al., 2005a). Symplasmic phloem unloading is a permanent feature of grass stems where barriers in anticlinal walls of bundle sheath cells block radial movement through their stem apoplasms (e.g., sugarcane—Jacobsen et al., 1992; wheat—Aoki et al., 2004; rice—Scofield et al., 2007).

IS SYMPLASMIC SIEVE ELEMENT UNLOADING DOMINATED BY BULK FLOW?

A key element of the high-pressure manifold model of phloem transport and partitioning is that SE unloading occurs as a bulk flow of phloem sap. Direct demonstration of bulk flow through STs has proved to be a technically challenging exercise. The most compelling evidence has been real-time detection of sharp fronts of fluorochromes moving through SEs and across sieve plates by confocal laser scanning microscopy (Knoblauch and van Bel, 1998) and concurrence of phloem transport velocities of water (Windt et al., 2006), and of radiolabelled solutes (Wardlaw, 1990). Plasmodesmal diameters (40–60 nm) and lengths (400–800 nm) preclude resolving solution flows through them by current confocal microscopy or MRI technologies. In the meantime, evaluating whether bulk flow occurs through plasmodesmal canals will depend upon innovative indirect measures and observations (e.g., Liesche and Schulz, 2012).

The sub-structure of plasmodesmata supporting cell-to-cell movement of solutes and water is complex and its precise architecture is at the limit of resolution by electron microscopy. Solute and water movement is considered to occur through the so-called cytoplasmic sleeve, the space located between the centrally-located desmotubule and plasma membrane lining the plasmodesmal outer boundary. Computer assisted tomography of electron microscope images suggest that plasmodesmal cytoplasmic sleeves are divided into 2–3 nm wide microchannels delimited by protein filaments that interconnect desmotubule and plasma membrane (Fisher, 2000). These dimensions correspond with selective cell-to-cell trafficking, between various cell types of ground tissues, of membrane-impermeant fluorochromes with molecular sizes up to 800 Da (Fisher, 2000). In contrast, larger sized fluorescently-tagged molecules (e.g., dextrans, ficoll—Fisher and Cash-Clark, 2000a; GFP alone or GFP-fusion proteins—Stadler et al., 2005a,b) have been found to move from SEs to vascular parenchyma cells in root apices (up to 67 kDa—Stadler et al., 2005a), sink leaves (up to 50 kDA—Stadler et al., 2005a) and developing seeds of wheat (up to 400 kDa—Fisher and Cash-Clark, 2000a) and Arabidopsis (up to 47 kDa—Stadler et al., 2005b). In all cases, subsequent movement from vascular parenchyma to ground parenchyma cells was found to be constrained to smaller molecular sizes—root apices (up to 27 kDa—Stadler et al., 2005a); developing seeds of wheat (up to 10 kDa—Fisher and Cash-Clark, 2000a) and Arabidopsis (up to 27 kDa—Stadler et al., 2005b). Overall these findings suggest that the SE/CC complexes and vascular parenchyma cells form one symplasmic domain, distinguishable from an interconnected ground tissue symplasmic domain with lower plasmodesmal permeabilities.

Deducing the molecular dimensions of plasmodesmal microchannels accounting for these two symplasmic domains is problematical on a number of grounds. These include possible plasmodesmal damage by high molecular weight fluorochromes (Fisher and Cash-Clark, 2000a) and by pressure-induced unfolding tertiary structures of macromolecules at plasmodesmal orifices with subsequent movement of the 'linearized' macromolecule through the cytoplasmic sleeve ('reptation' and for further information, see Proseus and Boyer, 2005). With these caveats in mind, observed rapid movement of microinjected fluorochromes of known Stokes radii (see above) indicate microchannel radii of 4–8 nm for phloem symplasmic domains and 1–2 nm for non-phloem symplasmic domains. Future

attempts to obtain estimates of plasmodesmal microchannel diameters could consider microinjecting conducting STs (Fisher and Cash-Clark, 2000a) with non-deformable colloidal gold particles of known Stokes radii (Proseus and Boyer, 2005).

Given the above estimates of plasmodesmal microchannel radii, the Hagen-Poiseuille Law (and see Equation 1) predicts that any symplasmic bulk flow of phloem sap exiting from SE/CC complexes will encounter considerable hydraulic resistances and hence a proportionate reduction in hydrostatic pressure upon entering recipient cells. These predictions are consistent with the presence of large (1.0 MPa) differences in hydrostatic pressures between release phloem SEs and cortical/epidermal cells of root tips measured directly using aphid stylet micromanometry and pressure probe respectively (**Table 2**). Similarly, large differences in cell sap osmotic potentials of SEs and adjacent cortical/epidermal cells (**Table 2**) reflect equally large differences in hydrostatic pressures as apoplasmic water potentials of these cells are identical in the absence any apoplasmic barriers and stable as a result of their hydraulic isolation from the remainder of the plant body (Lalonde et al., 2003). Radial bulk flow from release phloem SEs outward across various concentric layers of cell types would be expected to result in a progressive decline in cell hydrostatic pressures. This feature is apparent in estimates of cell osmotic potentials reported for maize root tips (Warmbrodt, 1987) as well as for pressure probe measurements of files of cortical cells in elongating *Ricinus* epicotyls (Meshcheryakov et al., 1992). Consistent with the high-pressure manifold model of phloem transport (**Figure 1**), the largest drop (ca 1.0 MPa) in osmotic and hydrostatic pressures occur between SEs and surrounding vascular parenchyma cells in root tips and developing wheat grains (**Table 2**).

A common, but largely untested generalization, is that the hydrodynamic radii of plasmodesmal microchannels prevent bulk flow by wall frictional drag spread throughout the breadth of the narrow water column. The data generated by Don Fisher's investigations of phloem unloading in developing wheat grains is sufficiently robust and complete (see Fisher and Cash-Clark, 2000a,b and references cited therein) to test whether plasmodesmal microchannel dimensions and densities are sufficient to support observed volume flow rates assuming that transport in these microchannels conform to the Hagen-Poiseuille Law (see Equation 1). Following the approach described by Fisher and Cash-Clark (2000a), the proposition was tested by determining whether sufficient numbers of plasmodesmata interconnecting SE/CC complexes with vascular parenchyma cells are present to support a known volume flow rate of 10 μL day^{-1} into a developing wheat grain across a set of microchannel radii (see **Table 3**). Briefly, volume flow rate through a single microchannel was computed (R_v – Equation 1) from which microchannel numbers supporting the observed volume flow rate were derived. From these data, microchannel numbers per plasmodesmata were estimated assuming that a single row of microchannels occupy 50% of the sleeve cross-section. Microchannel numbers per plasmodesma then allow determination of plasmodesmatal numbers required to support the observed volume flow rate (**Table 3**). Adequacy of microchannel radii were evaluated on the basis that estimated plasmodesmal numbers were equal to, or less than, observed plasmodesmal numbers interconnecting SE/CC complexes with vascular parenchyma cells—4.4×10^7 per grain (Wang et al., 1995). These comparisons show that for the smallest Stokes radius of 0.5 nm, the observed pressure differential of 1.0 MPa (Fisher and Cash-Clark, 2000b) is not adequate to support the observed volume flow rate (**Table 3**). However, radii greater than this contribute spare capacity so that at 1 nm, hydrostatic pressure differentials could be lowered to 0.4 MPa before volume flow rates were compromised. Comparing our approach with that of Fisher and Cash-Clark (2000a) for a microchannel radius of 10 nm yielded identical findings of 10×10^4 plasmodesmata (see **Table 3**) were required to support the observed volume flow rate driven by a hydrostatic pressure differential of

Table 2 | Osmotic or hydrostatic pressures of sieve tubes and adjoining specified cells in root tips and developing wheat grains.

Pressure potential (MPa)	Root-tip cell type:		Pressure difference (MPa)	Plant species	References
	Sieve element	Elongating epi/ cortical cell			
Osmotic	−1.62	−0.98	−0.64	Maize	Warmbrodt, 1987
Osmotic	−1.42	−0.71	−0.71	Barley	Prichard, 1996
Turgor					
High-K plants	1.62	0.33	1.29	Barley	Gould et al., 2004a
Low-K plants	1.32	0.32	1.0		
Pressure potential (MPa)	**Grain cell type:**		**Pressure difference (MPa)**	**Plant species**	**References**
	Sieve element	**Vascular parenchyma**			
Turgor				Wheat	Fisher and Cash-Clark, 2000b
Normal watered	1.11	0.12	1.0		
Water-stressed	1.30	0.08	1.12		

Adapted from Patrick (2012).

Table 3 | Impact of differing plasmodesmal (pd) microchannel radii[a], predicted by the Hagen-Poiseuille Law (*see* Equation 1), on plasmodesmal numbers required to support the observed volume flow rate (R_v)[b] of phloem sap, of specified viscosity[c], symplasmically unloaded from SE/CC complexes to vascular parenchyma cells of developing wheat grains at a transcellular hydrostatic pressure differential of 1.0 MPa across the interconnecting plasmodesmata of known length[d].

Microchannel radius (nm)	Predicted[e] R_v per microchannel ($\times 10^4$ nm^3 s^{-1})	Microchannel numbers supporting observed R_v ($\times 10^4$)[f]	Microchannel numbers per pd[g]	Pd numbers supporting observed R_v ($\times 10^3$)[h]
0.5	2.46	472,313	33	143,125
1.0	39.3	29,524	17	17,367
1.5	199	5832	11	5302
2.0	629	1845	8	2306
4.0	10,057	115	4	288
8.0	160,916	7	2	35.0
10.0	392,900	3	1	10.0

Plasmodesmal number estimates are compared with an observed value for developing wheat grains at the SE/CC complex and vascular parenchyma cell interface of 44,000 × 10^3 per grain (Wang et al., 1995).

[a] Estimates of microchannel radii of plasmodesmata (for details, see Section Is Symplasmic Sieve Element Unloading Dominated by Bulk Flow?)

[b] Observed by volume flow rate (R_v) of 11.6 × 10^{13} nm^3 s^{-1} (Fisher, 1990).

[c] Averaged phloem sap viscosity of 2 × 10^{-9} MPa s (Mullendore et al., 2010).

[d] Cell wall thickness and hence plasmodesmatal length between SE/CC complexes and vascular parenchyma cells in developing wheat grains—500 nm (Fisher and Cash-Clark, 2000a).

[e] R_v predicted using the nominated data sets in the Hagen-Poiseuille Law (Equation 1).

[f] Microchannel numbers supporting observed R_v derived as the ratio of observed by volume flow[a] to predicted flow rate per microchannel[e].

[g] Microchannel numbers per plasmodesmata were estimated assuming that their equators (diameters) are positioned on a circumference of half the internal radius (i.e., 10.5 nm—Wang et al., 1995) of plasmodesmata interconnecting SE/CC complexes to vascular parenchyma cells and that they occupy 50% of this circumference.

[h] Plasmodesmal numbers supporting the observed R_v derived as the ratio of microchannel numbers supporting observed R_v[f] and microchannel numbers per plasmodesmata[g].

1.0 MPa. Overall, these analysis demonstrate that known numbers of plasmodesmata interconnecting SE/CC complexes with adjacent vascular parenchyma of developing wheat grains can support observed volume flow rates if their microchannel radii are 1 nm or greater. Significantly, this conclusion can be extended to root tips as the observed hydrostatic pressure differences (**Table 2**) are of a comparable magnitude to model based predictions required to account for symplasmic transport by bulk flow (Brete-Harte and Silk, 1994).

If bulk flow is the primary transport mechanism by which resources are symplasmically unloaded from release phloem, it follows that rates of phloem unloading should respond proportionately to alterations in hydrostatic pressure differences between SEs and recipient sink cells. Consistent with this proposition is the finding that exposing root tips (Schulz, 1994; Pritchard et al., 2000) or abraded portions of mature stems, known to be unloading symplasmically (Patrick and Offler, 1996), to solutions containing non-permeating osmotica (mannitol and polyethylene glycol-8000 respectively), increased accumulation of phloem imported ^{14}C-photoassimilates at the osmoticum-exposed sites. Since polyethylene glycol-8000 does not penetrate cell walls (Carpita et al., 1979), water withdrawal is restricted to the outer cortical cells. As a consequence, a presumptive radial gradient in turgor pressure (see Meshcheryakov et al., 1992) must be increased to account for enhanced bulk flow of phloem sap from SEs to cortical cells (Patrick and Offler, 1996). Linkage between cortical cell turgors and phloem unloading rates have been verified by real-time monitoring of cortical cell turgor and ^{11}C-photoassimilate import prior to, during and following exposure of maize root tips to mannitol (Pritchard et al., 2005). Similarly, attenuating hydrostatic pressure differentials have been shown to retard phloem unloading rates of photoassimilates. For instance, potassium deficiency slowed phloem unloading in barley root tips and this response was linked with a depression of SE hydrostatic pressures whilst cortical cell pressures remained unaltered (Gould et al., 2004a and see **Table 2**). Conversely exposure of maize root tips to galactose dissipated the radial hydrostatic pressure gradient by increasing cortical cell turgors causing a proportionate decrease in photoassimilate unloading (Pritchard et al., 2004).

Since SE/CC complexes and vascular parenchyma cells share identical apoplasmic water potentials, turgor differentials arise from intracellular osmotic differentials and, as a result, concentration differences of phloem sap constituents. Hence diffusion must be a component of phloem unloading and the issue of the relative contributions of symplasmic phloem unloading by diffusion and bulk flow becomes one of degree. Diffusion rates (R_i) of a solute i are described by Fick's First Law of diffusion as:

$$R_i = DA\Delta C/\Delta x \quad (3)$$

where D is the diffusion coefficient of solute i; A is the cross-sectional area ($r = \pi r^2$) of the diffusional pathway; ΔC is the

concentration difference of solute *i* between either end of the diffusive pathway; Δx is the length of the diffusive pathway.

To explore the potential roles of diffusion (Equation 3) and bulk flow (Equation 1) in SE unloading, published sucrose concentrations in SEs (Fisher and Gifford, 1986) and vascular parenchyma cells (Fisher and Wang, 1995) of developing wheat grains were used to estimate rates of diffusion and bulk flow through a range of microchannel radii examined in **Table 3**. The findings show that rates of diffusion and bulk flow were comparable for transport through a plasmodesmal microchannel radius of 2 nm for the wheat grain conditions (**Table 4**). Lesser radii increasingly favored diffusion whilst larger ones increasingly favored bulk flow. If the caveats around using intercellular movement of fluorescently-tagged macromolecules to obtain valid measures of plasmodesmal microchannel radii do not apply (see Section Is Symplasmic Sieve Element Unloading Dominated by Bulk Flow?), then this outcome fits nicely with symplasmic unloading from SEs through plasmodesmal microchannels, with radii in the range of 4–8 nm, being dominated by bulk flow (**Table 4**). As a corollary to this conclusion plasmodesmal microchannel radii of these dimensions may ensure phloem unloading occurs by bulk flow suggesting that this mechanism offers a selective advantage over diffusion in phloem unloading (for further discussion, see Section Does Bulk Flow Offer Advantages over Diffusion to Symplasmic Sieve Element Unloading?).

Currently, there is only limited, and indirect, experimental evidence that offers a means of distinguishing between diffusion and bulk flow as mechanisms for phloem unloading. For instance, distribution of unloaded ^{14}C-photoassimilates imported into coats of developing pea seeds occurs into distinct sectoral patterns (Grusak and Minchin, 1988). This is consistent with unloading by bulk flow rather than by diffusion that would result in ^{14}C-photoassimilates being dispersed equally in all directions. Similarly, correspondence of relative accumulation patterns of symplasmically unloaded non-metabolizable substrates (^{14}C-L-glucose and fluorescein) with that of ^{14}C-sucrose along elongating pea epicotyls, independent of their differing symplasmic concentration gradients, suggests symplasmic unloading of these disparate solutes is primarily by bulk flow rather than diffusion (Gougler Schmalstig and Cosgrove, 1990).

Table 4 | Comparison of estimated transport rates of sucrose by diffusion and by bulk flow through a specified range of plasmodesmal microchannel radii each with a length of 500 nm for reported sucrose concentrations (C) in SEs[a] and vascular parenchyma cells[b] of developing wheat grains.

Microchanne radius (nm)	Transport rates ($\times 10^{-10}$ nmol s^{-1}) by:			
	Diffusion at a ΔC (mM) of:		Bulk flow at a C (mM) of:	
	200	400	450	600
0.5	1.64	3.28	0.22	0.14
1.0	6.53	13.1	1.77	2.36
1.5	14.7	29.4	8.95	11.9
2.0	26.2	52.3	28.2	37.7
4.0	105	209	453	603
8.0	418	837	7,241	9,655

*Rates of sucrose diffusion were estimated using Fick's First Law[c] (Equation 3) for two trans-plasmodesmal differences in sucrose concentration (ΔC). Bulk flow rates of sucrose were estimated as the product of estimated volume flow rates (see **Table 3** and Equation 1) and SE sucrose concentrations (C)[a].*

[a] Sucrose concentrations of SE sap collected from exuding pedicels of developing wheat grains ranged from 450 to 600 mM (Fisher and Gifford, 1986).

[b] Sucrose concentrations detected in frozen tissue slices of developing wheat grains containing vascular parenchyma cells ranged from 200 to 260 mM (Fisher and Wang, 1995). These concentrations are assumed to be representative of cytosolic sucrose concentrations in vascular parenchyma cells abutting SE/CC complexes. This assumption is an approximation as it respectively accepts that cytosolic and vacuolar sucrose concentrations are in equilibrium and that the sucrose concentration in all vascular parenchyma cells is identical.

[c] $D_{sucrose}$ is 0.52 $\times$ 10^9 nm^2 s^{-1} in water at 25 °C. ΔC values are derived from differences between SE[a] and vascular parenchyma[b] sucrose concentrations.

DOES BULK FLOW OFFER ADVANTAGES OVER DIFFUSION TO SYMPLASMIC SIEVE ELEMENT UNLOADING?

The most obvious answer to this question is embedded in a comparison of the Hagen-Poiseuille Law for bulk flow (Equation 1) and Ficks First Law of diffusion (Equation 3). That is, rates of these two processes are proportional to the radius of the conducting pipe raised to the fourth and second powers respectively. Thus, control of pipe radius can exert a four-fold greater control over rates of bulk flow compared to diffusion. This translates into an operational system whereby modest changes in microchannel dimensions can elicit substantial impacts on bulk flow rates of solute unloading from release phloem as illustrated in **Tables 3** and **4**.

In addition to optimizing control over phloem unloading (see above), bulk flow also avoids a dependence upon a very high level of co-ordination of unloading between individual solute species to avoid compromising phloem transport as outlined in the following scenario. If symplasmic unloading were to occur primarily by diffusion, then a mechanism must be present to ensure exit rates of each solute species and water from release phloem SEs match their rates of replenishment by axial transport. This is not a trivial requirement as diffusion rate of each solute moving through the same unloading pathway geometry depends not only on their concentration differences between SEs and vascular parenchyma cells but also on their individual diffusion coefficients (see Equation 3). Moreover phloem-imported water will diffuse at rates dictated by water potential differences between SE-CCs and vascular parenchyma cells. In these circumstances it would be very problematic for axial rates of each solute and water transported into, to match those of their diffusional exit from, release phloem SEs. As a consequence, regulation of ST hydrostatic pressures of release phloem could be compromised. This problem does not apply if SE unloading were to occur by bulk flow since solutes and solvent are transported at identical fluxes (i.e., velocities).

HYDRAULIC CONDUCTANCES, NOT HYDROSTATIC PRESSURE DIFFERENTIALS, REGULATE SYMPLASMIC UNLOADING

Studies using root tips currently provide the most information on which to evaluate relative regulatory influences of plasmodesmal

hydraulic conductances (i.e. the manifolds—see **Figure 1** and Equation 2) and hydrostatic pressure differentials (Equation 1) on phloem unloading. Axial profiles of cortical cell turgors along roots of a number of plant species have been found to be invariant compared to a distinct axial profile (commencing at the root tip) of accelerating and de-accelerating zones of extension growth and hence solute import rates (Pritchard et al., 2000). The accelerating zone of root extension is symplasmically coupled with release phloem whilst the de-accelerating extension zone may (Stadler et al., 2005a) or may not be symplasmically-coupled (Pritchard et al., 2005) depending upon prevailing source/sink ratios (Patrick and Offler, 1996). Absence of any inverse relationship between accelerating rates of root extension (a measure of R_v and see Equation 1) and cortical cell turgor points to hydraulic conductances of plasmodesmata, located along the symplasmic unloading pathway, exerting a major regulatory influence over radial fluxes of water and hence solutes travelling from SEs to cortical cells. This explanation also accounts for transport behaviors in developing seeds whereby coat cell turgors were found to be invariant across a three-fold range of seed growth rates exhibited between French bean cultivars (Thomas et al., 2000).

Manipulating extension growth of, and hence resource import into, root tips can reproduce the above findings. For instance, acid-induced increases in root extension rates were not accompanied by any change in cortical cell turgors (Winch and Pritchard, 1998). Similarly, a step 20°C decrease in root tip temperature elicited an immediate slowing of photoassimilate import whilst cortical cell turgors remained unaffected (Pritchard et al., 2000). In addition, responses of resource unloading to these experimental perturbations were rapid (Winch and Pritchard, 1998; Pritchard et al., 2000) suggesting that any alterations in plasmodesmal hydraulic conductances likely result from plasmodesmal gating rather than *de novo* plasmodesmal formation.

The proposed role of plasmodesmal hydraulic conductances in regulating phloem unloading does not preclude shifts in hydrostatic pressure differentials between SEs and cortical cells exerting an influence such as demonstrated for potassium deficiency (Gould et al., 2004a) and decreasing extensibility of root tip cells following exposure to galactose (Pritchard et al., 2004). Therefore, there is a need to accommodate hydrostatic pressure differentials and plasmodesmal hydraulic conductances into a unified regulatory scheme. One possible scenario is hydrostatic pressure differentials exert a course control around which plasmodesmal hydraulic conductance elicit fine control. This concept is supported as follows. Since hydrostatic pressure differentials between SEs and surrounding cells represent more than 80% of SE hydrostatic pressures (**Table 2**), there is only modest scope for increasing hydrostatic pressure differentials and reductions will depend upon major decreases in SE, or increases in sink cell, hydrostatic pressures. Thus, at best, alterations in hydrostatic pressure differentials could impose course control over resource unloading. In contrast, for a given hydrostatic pressure differential, the Hagen-Poiseuille Law (see Equation 1) predicts that relatively small alteration in hydrodynamic radii of plasmodesmal microchannels will exert considerable fine control over their hydraulic conductances (radius to the fourth power) determining bulk flow or their permeabilities (radius raised to the second power—see Equation 3) for diffusion.

Control of phloem unloading by plasmodesmal hydraulic conductances could be exerted at two levels. First, plasmodesmal geometries (widths; lengths—Equation 2) and densities, formed during their development at various cellular interfaces located along phloem unloading pathways set upper limits for symplasmic hydraulic conductances. For example, inferred from Stoke's radii of the permeating molecules, these data yield conservative estimates of plasmodesmal microchannel radii for phloem and non-phloem domains that extend over a two-fold range (see Section Is Symplasmic Sieve Element Unloading Dominated by Bulk Flow?). In relative terms, these radii translate into a 16-fold range of hydraulic conductances. Second, plasmodesmal gating (open versus closed), mediated by callose deposition/hydrolysis around plasmodesmal neck regions, has been demonstrated to play a key role in regulating transmission of macromolecular signals orchestrating cell development (Burch-Smith and Zambryski, 2012). Plasmodesmal gating by callose deposition/removal also appears to apply to the transport of small molecules by the rapid responses to exposure of root tips to hydrogen peroxide (Rutschow et al., 2011). In addition, when root tips of hydroponically-grown pea seedlings were exposed to hyperosmotic conditions, plasmodesmal neck constrictions disappeared and their cytoplasmic sleeved expanded within 1 h of transfer to solutions containing 350 mM mannitol (Schulz, 1995). Significantly, these alterations in plasmodesmal canal geometries coincided with a three-fold increase in phloem unloading rates at the root tips (Schulz, 1994).

HOW IS SINK DEMAND COMMUNICATED TO REGULATE PLASMODESMAL CONDUCTANCES?

Conductance for symplasmic movement between two adjacent cells is a function of their averaged conductance of plasmodesmata summed over the density of plasmodesmata located in their shared wall. Plasmodesmal densities are under both positional and developmental control (Burch-Smith and Zambryski, 2012). Regulatory mechanisms controlling plasmodesmal densities are beginning to be discovered and these exert a predictable positive influence on overall symplasmic conductance (e.g., Burch-Smith and Zambryski, 2010; Xu et al., 2012). For root apices, plasmodesmal densities in transverse walls are higher than those in periclinal walls and both densities progressively decrease with distance from the vascular tissues (Zhu et al., 1998). This plasmodesmal network possibly contributes to canalizing longitudinal flow of solutes from the phloem to root apical meristems (Stadler et al., 2005a). A similar scenario likely applies to shoot apical meristems. In contrast, a distinguishing feature of symplasmic unloading pathways servicing expansion/storage sinks is that plasmodesmal densities are least at their SE-CC/phloem parenchyma cell interfaces (Patrick and Offler, 1996). The bottleneck imposed by plasmodesmal densities at the SE-CC/phloem parenchyma cell interface is consistent with it being a major control point for phloem unloading (and also see Sections Axial Hydraulic Conductances of Sieve Tubes are Considerably Higher than Those of Phloem Unloading Pathways and Symplasmic Phloem Unloading by Bulk Flow Through a Plasmodesmal Manifold). This feature focuses

attention on hydrodynamic radii of plasmodesmal canals at this interface as a major influence over symplasmic conductance (Section Symplasmic Phloem Unloading by Bulk Flow Through a Plasmodesmal Manifold and **Tables 3** and **4**). How plasmodesmal conductances may be co-ordinated with temporal shifts in downstream sink demand can be speculated upon from an emerging understanding of how plasmodesmal conductances are regulated. This question is explored separately for meristematic and expansion/storage sinks.

Within meristematic sinks, plant hormones regulate rates of cell division and hence sink demand. This hormone function is linked with their ability to directly act on transport processes delivering resources to hormone-enriched sites (Patrick, 1987). Fully-elongated bean stems, following surgical removal of acropetally growing regions and treating the decapitated stem stumps with plant hormones reproduces the hormonal status of meristematic sinks, but with the absence of cell division/expansion and hence altered sink demand (Patrick, 1987 and literature cited therein). Using the decapitated stem model, in which differentials in sucrose concentration or cell turgor between stem SEs and cortical cells were clamped experimentally, demonstrated that cytokinins and gibberellins stimulated phloem import of photoassimilates by increasing plasmodesmal permeabilities or conductances respectively along the symplasmic-unloading pathway at the site of hormone application (Patrick and Offler, 1996). How hormones exert their putative control of plasmodesmal permeabilities/conductances is unknown, but the rapid (h) and step change in rates of photoassimilate import by stems treated with gibberellin or cytokinin (Mulligan and Patrick, 1979; Turvey and Patrick, 1979) is consistent with post-translational regulation. Rapid (h) effects on plasmodesmal permeabilities have been reported in Arabidopsis root tips exposed to hydrogen peroxide (Rutschow et al., 2011) providing a regulatory pathway through which hormones (De Tullio et al., 2010) may or may not act on plasmodesmal conductances. At this juncture, hormonal control of plasmodesmal permeabilities/conductances awaits independent verification (cf. Burch-Smith and Zambryski, 2012 and see Rutschow et al., 2011), but offers an attractive model of matching resource delivery with resource demand by meristematic cells. Based on observations in root apices (see Section Axial Hydraulic Conductances of Sieve Tubes are Considerably Higher than Those of Phloem Unloading Pathways), the primary site at which hormonal control is likely to be exerted over bulk flow rates is on the combined hydraulic conductances of plasmodesmata and differentiating sieve pores located in the provascular strands canalizing an axial flow of resources toward the root meristem (and see **Figure 2**). In addition, hormonal regulation of protophloem formation from provascular strands (Aloni, 2010) is accompanied by coalescing pit fields of plasmodesmata into sieve pores with radii several orders larger than those of plasmodesmal microchannels thus conferring substantial increases in hydraulic conductances of axial transport pathways (see Equation 2).

For expansion/storage sinks, some insights into the regulatory mechanisms integrating sink demand with plasmodesmal control of resource flow along phloem unloading pathways has been obtained from observations of developing seeds. Phloem unloading pathways in developing seeds universally contain a symplasmic discontinuity at, or proximal to, their maternal and filial interfaces at which phloem-imported resources are released to, and retrieved from, the seed apoplasmic space (Zhang et al., 2007a and see **Figure 3**). A hint that hydraulic conductances of

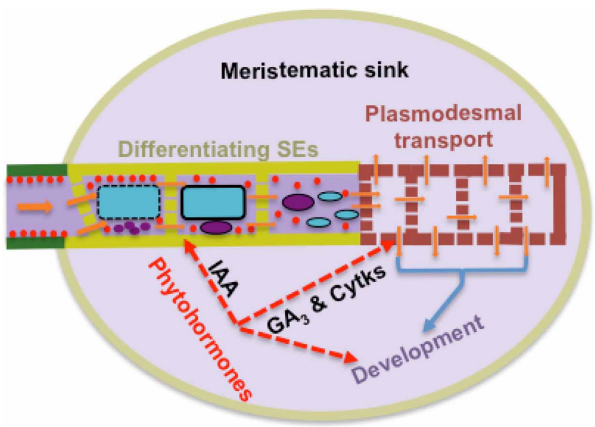

FIGURE 2 | Model of a high resistance pathway encountered by resource flow from transport phloem (green) into meristematic sinks through protophloem SEs differentiating from provascular cells (khaki) arranged in series with symplasmic movement through plasmodesmata of meristematic cells (brown). One tier of sink control of resource import is mediated by phytohormones integrating hydraulic conductances of this transport pathway with sink demand as illustrated for indole-3-actic acid (IAA), gibberellic acid (GA_3) and cytokinins (Cytks). During differentiation, each developing SE forms a large central vacuole (blue) and conspicuous protein bodies and plastids (red). Their vacuole and nucleus (purple) finally degrade to leave a parietal cytoplasm of protein bodies and plastids. Co-incidentally pit fields of plasmodemata coalesce into sieve pores.

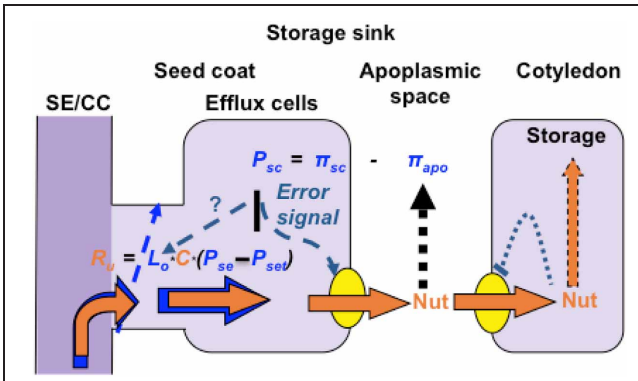

FIGURE 3 | Turgor homeostasis model of phloem unloading in developing seeds of grain legumes. Sucrose uptake by, and storage, in cotyledons is coupled through a negative feedback transcriptional regulation of sucrose transporter activity mediated through intracellular sucrose levels. Activities of seed coat sucrose effluxers are coordinated with cotyledon demand by a turgor-homeostat mechanism that osmotically (π) detects alterations (error signal) in apoplasmic sucrose pool sizes as a deviation from a turgor (*P*) set point. Symplasmic unloading from release phloem SE/CC complexes by bulk flow is regulated by plasmodesmal hydraulic conductances (L_o) under control of the turgor homeostat functioning as a central control hub to regulate resource flow from SE/CC complexes to ultimate storage in cotyledons.

plasmodesmata, forming the maternal component of the symplasmic unloading pathway (**Figure 3**), regulate resource flows is the finding that a pharmacological block of sucrose uptake into endosperm of attached wheat grains was not accompanied by any change in sucrose concentrations located in cells forming the phloem unloading pathway in maternal grain tissues (Fisher and Wang, 1995). The absence of continued phloem unloading into, and accumulation of sucrose by, maternal grain tissues suggests that plasmodesmata linking SEs to resource release sites in the nucellus projection (Wang et al., 1995) must have immediately gated closed upon inhibiting sucrose uptake into the endosperm. The response points to a direct link between sucrose uptake by endosperm and plasmodesmal conductances at SE-CC-vascular parenchyma interfaces. This proposed relationship accounts for the observed invariance of sucrose concentrations, located at various points along phloem-unloading pathways in developing seeds, when sucrose fluxes are altered (Fader and Koller, 1985; Thomas et al., 2000). The mechanism responsible for adjusting plasmodesmal conductances at SE-CC/phloem parenchyma cell interfaces with activities of membrane transporters responsible for resource transit to, and from, seed apoplasmic spaces is not known. However, a reasoned case can be made for cell turgor integrating these disparate transport processes based on a turgor-homeostat mechanism that co-ordinates resource release to, and uptake from, seed apoplasmic spaces (Zhang et al., 2007a and see **Figure 3**). Cell turgor-regulated control of plasma membrane transporter activities located in seed maternal tissues is mediated through mechano-sensitive properties of the actin cytoskeleton detecting subtle turgor-induced changes in cell volume (Zhang et al., 2007b). Since components of the actin cytoskeleton form part of the plasmodesmal canal sub-structure and actin dynamics can influence plasmodesmal permeabilities (White and Barton, 2011), it then follows that plasmodesmal conductances at the SE-CC/phloem parenchyma cell interface may be co-ordinately regulated with activities of membrane transporters to meet alterations in resource demand by filial tissues of developing seeds. In addition, actin-dependent control of plasmodesmal hydraulic conductances, through a turgor-homeostatic mechanism, likely operates to regulate phloem unloading of resources during the pre-storage phase of seed development. To directly test this proposed mechanism experimentally will not be a trivial exercise.

THE HIGH-PRESSPURE MANIFOLD MODEL AND CROP BIOMASS YIELD POTENTIAL

Crop biomass yield potential is the harvested yield per plant of any cultivar raised under optimal environmental conditions for its development in the absence of any limitation imposed by abiotic or biotic stresses (Evans and Fischer, 1999). This yield parameter has proven to be a reproducible and robust selection tool in conventional breeding programs for crop yield realized at the farm gate (Fischer and Edmeades, 2010). Components of crop biomass yield are the number of harvested units (seed, fruit, tubers etc.) per plant and the average biomass per harvested unit. Numbers of harvested units per plant are determined during the meristematic (pre-storage) phase of yield organ development. For example, at fruit and seed set, their relative levels of loss through abortion largely govern their numbers at harvest. In addition, longevity of the meristematic phase determines cell numbers of each harvestable unit and hence their harvested size. Both abortion and meristematic activity are very sensitive to abiotic stresses (Ruan et al., 2012). Cessation of meristematic activity is replaced by cell expansion and accumulation of storage product(s)—the so-called storage phase of harvestable organ development during which crop biomass yield potential, set during the pre-storage phase, is realized.

The elements carbon, hydrogen and oxygen account for ca 90% of crop biomass yield. These elements reach the harvested units as phloem-imported sugars that form the major osmotica generating ST hydrostatic pressures propelling bulk flow from source to sink. The preceding analysis of the high-pressure manifold model of phloem transport identified hydraulic conductances of plasmodesmata, located at the sink-end of phloem pathways, as key regulators of bulk flow rates from source to sink and for partitioning of flow rates between competing sinks (**Figure 1**). This should position the high-pressure manifold model of phloem transport as a core determinant of crop biomass yield potential; a claim yet to be verified experimentally. However, as reviewed below, there are hints, based on circumstantial evidence, that hydraulic conductances of phloem unloading pathways play a central role in determining harvested organ numbers per plant as well as influencing their rates of storage product accumulation and hence their harvested biomass.

The pre-storage phase of harvestable organ development is photoassimilate limited as demonstrated by positive responses of harvestable organ numbers to elevated source/sink ratios (Ruan et al., 2012). Interpreted in terms of the high-pressure manifold model of phloem transport, increased source output of photoassimilates ultimately results in elevated sucrose concentrations accumulating in, and hence raising hydrostatic pressures of, the collection and transport phloem. This would result in increased hydrostatic pressure differentials forming across the low hydraulic conductance axial pathways of developing provascular stands linking the transport phloem with meristems of developing harvested organs (Section Conditions for High Hydrostatic Pressures Throughout Sieve Tube System With Minimal Gradients from Source to Sink). As a consequence, bulk flow rates of phloem sap increase into these tissues as shown by altering ST hydrostatic pressure potentials of release phloem and ground cells of root tips (Gould et al., 2004a; Pritchard et al., 2004). However, elevating crop photosynthesis as a yield improvement strategy at the pre-storage phase of development can be questioned on efficiency grounds since meristem carbon demand is considerably less than whole plant photoassimilate production. In addition, harvest unit abortion appears to be regulated by sugar signals averting expression of programmed cell death genes and depressing genes regulating cell division (Ruan et al., 2012). These regulatory events would require only small increases in photoassimilate import into pre-storage sinks. In this context, altering hydraulic conductances of axial pathways of developing provascular strands provides a sink specific action on resource flows and does not encounter a carbon cost. As outlined in Section How Is Sink Demand Communicated to Regulate Plasmodesmal Conductances?, plant hormones could function in this way (see **Figure 2**). Consistent with this

proposition, transgenic upregulation of hormonal levels in harvestable units during their pre-storage phase of development has been found to result in increasing numbers of harvestable units. For example increased numbers of seeds (Radchuk et al., 2012), fruit (Bartrina et al., 2011) and tubers (Prat, 2010) per plant. For developing seeds, another layer of control could be exercised by sink demand of their filial tissues sensed through a turgor homeostatic mechanism that adjusts plasmodesmal hydraulic conductances of phloem unloading pathways delivering resources to these sinks (see **Figure 3** and associated text in Section How Is Sink Demand Communicated to Regulate Plasmodesmal Conductances?). This scenario is illustrated by transgenically expressing a post-translationally de-regulated *Zea mays* ADP-glucosepyrophosphorylase, under control of an endosperm-specific promoter, in pre-storage grains of wheat (Smidansky et al., 2002), rice (Smidansky et al., 2003) and maize (Hannah et al., 2012). The transgenic plants supported 36, 19 and 23 percent increases respectively in grain numbers per plant and hence their biomass yields without compromising size (biomass) of their individual grains. Interestingly, increased sink demand of the developing seeds led to a de-repression of leaf photosynthesis in wheat (Smidansky et al., 2007) consistent with spare phototosynthetic potential during the pre-storage phase of seed development.

The storage phase filling rates are characteristically sink-limited (Borrás et al., 2004). The low hydraulic conductances of plasmodesmata interconnecting SE-CC complexes with adjacent phloem parenchyma cells in the release phloem of storage phase sinks could well account for a component of their sink limitation (see Section How Is Sink Demand Communicated to Regulate Plasmodesmal Conductances?). For example, in order to generate a 10% increase in bulk flow rates through these plasmodesmata in storage-phase wheat grain would require depressing phloem parenchyma cell hydrostatic pressure to zero (see **Table 2**). In contrast, the turgor-homeostat linkage between filial sink demand and plasmodesmal hydraulic conductances (**Figure 3** and see Section How Is Sink Demand Communicated to Regulate Plasmodesmal Conductances?) readily could be accommodated by increasing radii of their plasmodesmal canals by 1.3% (**Table 3**) as predicted by the Hagen-Poiseuille Law (see Equation 1). That such an outcome is achievable has been demonstrated by transgenic increases in sucrose transporter activity of filial tissues of developing pea (Rosche et al., 2002) and wheat (Weichert et al., 2010) seeds increasing seed biomass gains by 23 and 16 percent respectively under controlled environmental conditions.

Overall, the above circumstantial evidence indicates that opportunities may well exist to improve crop biomass yield through manipulating resource transport informed by the high-pressure manifold model of phloem transport.

CONCLUSIONS

Reviewing current knowledge of phloem transport leads to a strong circumstantial case being made for the high-pressure manifold model of phloem transport. This is considered sufficient to warrant further study of the model. Initially, most effort could be directed to increasing knowledge of the plasmodesmal substructures and how these influence resource flow through their plasmodesmal microchannels. In particular, it is crucial to obtain direct evidence that hydraulic conductances of plasmodesmata, linking SEs with surrounding phloem parenchyma cells, support SE unloading of phloem sap by bulk flow. This enterprise could be informed by the exciting findings emerging from investigations of water flows through nanotubes showing that, with radii of several nm, flow velocities exceed those predicted by the Hagen-Poiseuille law by four to five orders of magnitude (Majumber et al., 2005). The ensuing knowledge base should deliver novel opportunities to discover how plasmodesmal hydraulic conductances are regulated to match sink demand for resources. Incorporating these putative findings into re-working models of phloem transport (cf. Thompson and Holbrook, 2003; Jensen et al., 2011, 2012) are anticipated to make substantial contributions that will allow behaviors of phloem transport to be predicted including identifying mechanisms regulating resource partitioning. The legacy left by Don Fisher's high-pressure manifold model is ripe for productive investigation that will undoubtedly deliver substantial conceptual advances to the field of phloem transport biology and innovative approaches to increasing crop yield potential.

DEDICATION

This paper is dedicated to the memory of Donald Fisher, a modest, but passionate, plant scientist. Don's contributions to advancing our conceptual knowledge of phloem transport biology were substantial, unyielding in their rigor and trail blazing in their impact on the field.

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REFERENCES

Aloni, R. (2010). "The induction of vascular tissues by auxin," in *Plant Hormones. Biosynthesis, Signal Transduction, Action!* ed P. J. Davies (Amsterdam: Springer), 485–518. doi: 10.1007/978-1-4020-2686-7_22

Aoki, N., Scofield, G. N., Wang, X.-D., Patrick, J. W., Offler, C. E., and Furbank, R. T. (2004). Expression and localisation of the wheat sucrose transporter TaSUT1 in vegetative tissues. *Planta* 219, 176–184. doi: 10.1007/s00425-004-1232–7

Bartrina, I., Otto, E., Strnad, M., Werner, T., and Schmülling, T. (2011). Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in Arabidopsis thaliana. *Plant Cell* 23, 69–80. doi: 10.1105/tpc.110.079079

Bieleski, R. L. (1966a). Accumulation of phosphate, sulphate and sucrose by excised phloem tissues. *Plant Physiol.* 41, 447–454. doi: 10.1104/pp.41.3.447

Bieleski, R. L. (1966b). Sites of accumulation in excised phloem and vascular tissues. *Plant Physiol.* 41, 455–466. doi: 10.1104/pp.41.3.455

Borrás, L., Slafer, G. A., and Otegui, M. E. (2004). Seed dry weight response to source-sink manipulation in wheat, maize and soybean: a quantitative re-appraisal. *Field Crops Res.* 86, 131–146. doi: 10.1016/j.fcr.2003.08.002

Brete-Harte, M. S., and Silk, W. K. (1994). Nonvascular, symplastic diffusion of sucrose cannot satisfy the carbon demands of growth in the primary root tip of Zea mays L. *Plant Physiol.*

105, 19–33. doi: 10.1104/pp.105.1.19

Burch-Smith, T. M., and Zambryski, P. C. (2010). Loss of INCREASED SIZE EXCLUSION LIMIT (ISE)1 or ISE2 increases the formation of secondary plasmodesmata. *Curr. Biol.* 20, 989–993. doi: 10.1016/j.cub.2010.03.064

Burch-Smith, T. M., and Zambryski, P. C. (2012). Plasmodesmata paradigm shift: regulation from without versus within. *Annu. Rev. Plant Biol.* 63, 239–226. doi: 10.1146/annurev-arplant-042811–105453

Carpita, N., Sabularse, D., Montezinos, D., and Delmer, D. P. (1979). Determination of the pore size of cell walls of living plant cells. *Science* 205, 1144– 147. doi: 10.1126/science.205.4411.1144

Chiou, T.-J., and Bush, D. R. (1998). Sucrose is a signal molecule in assimilate partitioning. *PNAS* 95, 4784–4788.

Daie, J., and Wyse, R. E. (1985). Evidence on the mechanism of enhanced sucrose uptake at low cell turgor in leaf discs of *Phaseolus coccineus*. *Physiol. Plant* 64, 547–552.

Deeken, R., Geiger, D., Fromm, J., Koroleva, O., Ache, P., Langenfeld-Heyser, R., et al. (2002). Loss of AKT2/3 potassium channel affects sugar loading into the phloem of Arabidopsis. *Planta* 216, 334–344. doi: 10.1007/s00425-002-0895–1

De Tullio, M. C., Jiang, K., and Feldman, L. J. (2010). Redox regulation of root apical meristem organization: connecting root development to its environment. *Plant Physiol. Biochem.* 48, 328e336. doi: 10.1016/j.plaphy.2009.11.005

Estruch, J. J., Peretó, J. G., Vercher, Y., and Beltrán, J. P. (1989). Sucrose loading in isolated veins of *Pisum sativum*: regulation by abscisic acid, gibberellic acid, and cell turgor. *Plant Physiol.* 91, 259–265. doi: 10.1104/pp.91.1.259

Evans, L. T., and Fischer, R. A. (1999). Yield potential: it definition, measurement and significance. *Crop Sci.* 39, 1544–1551. doi: 10.2135/cropsci1999.3961544x

Fader, G. M., and Koller, H. R. (1985). Seed growth rate and carbohydrate pool sizes of the soybean fruit. *Plant Physiol.* 79, 663–666. doi: 10.1104/pp.79.3.663

Fischer, R. A., and Edmeades, G. O. (2010). Breeding and cereal yield progress. *Crop Sci.* 50, S85–S98.

Fisher, D. B. (1990). Measurement of phloem transport rates by an indicator-dilution technique. *Plant Physiol.* 94, 455–462. doi: 10.1104/pp.94.2.455

Fisher, D. B. (2000). "Long-Distance Transport," in *Biochemistry and Molecular Biology of Plants*, ed B. Buchanan, W. Grissem, and R. Jones (Marylands: ASPB), 730–785.

Fisher, D. B., and Gifford, R. M. (1986). Accumulation and conversion of sugars by developing wheat grains: VI gradients along the transport pathway from the peduncle to the endosperm cavity during grain filling. *Plant Physiol.* 82, 1024–1030. doi: 10.1104/pp.82.4.1024

Fisher, D. B., and Wang, N. (1995). Sucrose concentration gradients along the post-phloem transport transport pathway in the maternal tissues of developing wheat grains. *Plant Physiol.* 109, 587–592 doi: 10.1104/pp.109.2.587

Fisher, D. B., and Cash-Clark, C. E. (2000a). Sieve tube unloading and post-phloem transport of fluorescent tracers and proteins injected into sieve tubes severed aphid stylets. *Plant Physiol.* 123, 125–137. doi: 10.1104/pp.123.1.125

Fisher, D. B., and Cash-Clark, C. E. (2000b). Gradients in water potential and turgor pressure along the translocation pathway during grain filling in normally watered and water-stressed wheat plants. *Plant Physiol.* 123, 139–147. doi: 10.1104/pp.123.1.139

Fondy, B. R., and Geiger, D. R. (1980). Effect of rapid changes in source-sink ratio on export and distribution of products of photosynthesis in leaves of *Beta vulgaris* L. and *Phaseolus vulgaris* L. *Plant Physiol.* 66, 945–949. doi: 10.1104/pp.66.5.945

Froelich, D. R., Mullendore, D. L., Jensen, K. H., Ross-Elliot, T. J., Anstead, J. A., Thompson, G. A., et al. (2011). Phloem ultrastructure and pressure flow: Sieve-Element-Occlusion-Related agglomerates do not affect translocation. *Plant Cell* 23, 4428–4445. doi: 10.1105/tpc.111.093179

Fu, Q., Cheng, L., Guo, Y., and Turgeon, R. (2011). Phloem loading strategies and water relations in trees and herbaceous plants. *Plant Physiol.* 157, 1518–1527. doi: 10.1104/pp.111.184820

Gil, L., Yaron, I., Shaliton, D., Sauer, N., Turgeon, R., and Wolf, S. (2011). Sucrose transport plays a role in phloem loading in CMV-infected melon plants that are defined as symplasmic loaders. *Plant J.* 66, 366–374. doi: 10.1111/j.1365-313X.2011.04498.x

Gougler Schmalstig, J., and Cosgrove, D. J. (1990). Coupling of solute transport and cell expansion in pea stems. *Plant Physiol.* 94, 1625–1633. doi: 10.1104/pp.94.4.1625

Gould, N., Thorpe, M. R., Minchin, P. E. H., Pritchard, J., and White, P. J. (2004a). Solute is imported to elongating root cells of barley as a pressure driven-flow of solution. *Funct. Plant Biol.* 31, 391–397. doi: 10.1071/FP03231

Gould, N., Minchin, P. E. H., and Thorpe, M. R. (2004b). Direct measurements of sieve element hydrostatic pressure reveal strong regulation after pathway blockage. *Funct. Plant Biol.* 31, 987–993. doi: 10.1071/FP04058

Gould, N., Thorpe, M. R., Koroleva, O., and Minchin, P. E. H. (2005). Phloem hydrostatic pressure relates to solute loading rate: a direct test of the Münch hypothesis. *Funct. Plant Biol.* 32, 1019–1026. doi: 10.1071/FP05036

Grignon, N., Touraine, B., and Durand, M. (1989). 5carboxyfluoroscein as a tracer of phloem translocation. *Am. J. Bot.* 76, 871–877.

Grusak, M. A., and Minchin, P. E. H. (1988). Seed coat unloading in *Pisum sativum*—osmotic effects in attached versus excised empty ovules. *J. Exp. Bot.* 39, 543–559. doi: 10.1093/jxb/39.5.543

Hammel, H. T. (1968). Measurement of turgor pressure and its gradients in the phloem of oak. *Plant Physiol.* 43, 1042–1048. doi: 10.1104/pp.43.7.1042

Hannah, L. C., Futch, B., Bing, J., Shaw, J. R., Boehlein, S., Stewart, J. D., et al. (2012). A shrunken-2 transgene increases maize yield by acting in maternal tissues to increase the frequency of seed development. *Plant Cell* 24, 2352–2356. doi: 10.1105/tpc.112.100602

Haupt, S., Duncan, G. H., Holzberg, S., and Oparka, K. J. (2001). Evidence for symplastic phloem unloading in sink leaves of barley. *Plant Physiol.* 125, 209–218. doi: 10.1104/pp.125.1.209

Hayes, P. M., Offler, C. E., and Patrick, J. W. (1985). Cellular structures, plasma membrane surface areas and plasmodesmatal frequencies of the stem of *Phaseolus vulgaris* L. in relation to radial photosynthate transfer. *Ann. Bot.* 56, 125–138.

Hu, L., Sun, H., Li, R., Zhang, L., Wang, S., Sui, X., et al. (2011). Phloem unloading follows an extensive apoplasmic pathway in cucumber (*Cucumis sativa* L.) fruit from anthesis to marketable maturing stage. *Plant Cell Environ.* 40, 743–748. doi: 10.1111/j.1365-3040.2011.02380.x

Hukin, D., Doering-Saad, C., Thomas, C. R., and Pritchard, J. (2002). Sensitivity of cell hydraulic conductivity to mercury is coincident with symplasmic isolation and expression of plasmalemma aquaporin genes in growing maize roots. *Planta* 215, 1047–1056. doi: 10.1007/s00425-002-0841–2

Jacobsen, K. R., Fisher, D. G., Maretzki, A., and Moore, P. H. (1992). Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Bot. Acta.* 105, 70–80.

Jensen, K. H., Lee, J., Bohr, T., Bruus, H., Holbrook, N. M., and Zwieniecki, M. A. (2011). Optimality of the Münch mechanism for the translocation of sugars in plants. *J. R. Soc. Interface* 8, 1155–1165. doi: 10.1098/rsif.2010.0578

Jensen, K. H., Liesche, J., Bohr, T., and Schulz, A. (2012). Universality of phloem transport in seed plants. *Plant Cell Environ.* 35, 1065–1076. doi: 10.1111/j.1365–3040.2011.02472.x

Kallarackal, J., and Milburn, J. A. (1984). Specific mass transfer and sink-controlled phloem translocation in castor bean. *Aust. J. Plant Physiol.* 10, 561–568.

Kempers, R., Ammerlaan, A., and van Bel, A. J. E. (1998). Symplasmic constriction and ultrastructural features of the sieve element/companion cell complex in the transport phloem of apoplasmically and symplasmically phloem-loading species. *Plant Physiol.* 116, 271–278. doi: 10.1104/pp.116.1.271

Knoblauch, M., and van Bel, A. J. E. (1998). Sieve tubes in action. *Plant Cell* 10, 35–50. doi: 10.1105/tpc.10.1.35

Körner, C. (2003). Carbon limitation in trees. *J. Ecol.* 91, 4–17. doi: 10.1046/j.1365–2745.2003.00742.x

Lalonde, S., Tegeder, M., Throne-Holst, M., Frommer, W. B., and Patrick, J. W. (2003). Phloem loading and unloading of amino acids and sugars. *Plant Cell Environ.* 26, 37–56 doi: 10.1046/j.1365–3040.2003.00847.x

Lee, D. R. (1981). Synchronous pressure-potential changes in the phloem of *Fraxinus americana* L. *Planta* 151, 304–308

Liesche, J., and Schulz, A. (2012). Quantification of plant cell coupling with three-dimensional photoactivation microscopy. *J. Microsc.* 247,

2–9. doi: 10.1111/j.1365–2818.2011.03584.x

Majumber, M., Chopra, N., Andrews, R., and Hinds, B. J. (2005). Enhanced flow in carbon tubes. *Nature* 438, 44. doi: 10.1038/43844a

Mason, T. G., and Maskell, E. J. (1928). Studies on the transport of carbohydrates in the cotton plant. II. The factors determining the rate and direction of movement of sugars. *Ann. Bot.* 42, 571–636.

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2006). Sink strength regulates photosynthesis in sugarcane. *New Phytol.* 171, 759–770. doi: 10.1111/j.1469–8137.2006.01785.x

Mencuccini, M., and Hölttä, T. (2010). The significance of phloem transport for the speed with which canopy photosynthesis and belowground respiration are linked. *New Phytol.* 185, 189–203. doi: 10.1111/j.1469–8137.2009.03050.x

Merchant, A., Peuke, A. D., Keital, C., MacFarlane, C., Warren, C. R., and Adams, M. A. (2010). Phloem sap and leaf d13C, carbohydrates, and amino acid concentrations in Eucalyptus globulus change systematically according to flooding and water deficit treatment. *J. Exp. Bot.* 61, 1758–1793. doi: 10.1093/jxb/erq045

Merchant, A., Buckley, T. N., Pfautsch, S., Turnbull, T. L., Samsa, G. A., Adams, M. A. (2012). Site-specific responses to short-term environmental variation are reflected in leaf and phloem-sap carbon isotopic abundance of field grown Eucalyptus globulus. *Physiol. Plant* 146, 448–459. doi: 10.1111/j.1399–3054.2012.01638.x

Meshcheryakov, A., Steudle, E., Komor, E. (1992). Gradients of turgor, osmotic pressure, and water potential in the cortex of the hypocotyl of growing Ricinus seedlings. *Plant Physiol.* 98, 840–852. doi: 10.1104/pp.98.3.840

Milburn, J. A., and Kallarackal, J. (1989). "Physiological aspects of phloem translocation," in *Transport of Photoassimilates*, ed D. A. Baker and J. A. Milburn (New York, NY: Lonhman Scientific and Technical Essex), 262–305.

Minchin, P. E. H., Thorpe, M. R., Farrar, J. F., and Koroleva, O. A. (2002). Source-sink coupling in young barley plants and control of phloem loading. *J. Exp. Bot.* 53, 1671–1676. doi: 10.1093/jxb/erf003

Mullendore, D. L., Windt, C. W., Van As, H., and Knoblauch, M. (2010). Sieve tube geometry in relation to phloem flow. *Plant Cell* 22, 579–593. doi: 10.1105/tpc.109.070094

Mulligan, D. R., and Patrick, J. W. (1979). Gibberellic-acid-promoted transport of assimilates in stems of *Phaseolus vulgaris* L. Localised versus remote site(s) of action. *Planta* 145, 233–238. doi: 10.1007/BF00454446

Münch, E. (1930). *Material Flow in Plants*. Translated 2003 by J. A. Milburn and K. H. Kreeb, Germany: University of Bremen; Jena Germany: Gustav Fischer Verlag.

Nie, P., Wang, X., Hu, L., Zhang, H., Zhang, J., Zhang, Z., et al. (2010). The predominance of the apoplasmic phloem-unloading pathway is interrupted by a symplasmic pathway during Chinese jujube fruit development. *Plant Cell Physiol.* 51, 1007–1018. doi: 10.1093/pcp/pcq054

Offler, C. E., and Horder, B. (1992). The cellular pathway of short distance transfer of photosynthates in developing tomato fruit. *Plant Physiol.* 99S, 41.

Pate, J. S., and Arthur, D. J. (2000). Uptake, partitioning and utilization of carbon and nitrogen in the phloem bleeding tree, Tasmanian blue gum (*Eucalyptus globulus*). *Aust. J. Plant Physiol.* 27, 869–884. doi: 10.1071/PP99149

Patrick, J. W. (1987). "Are hormones involved in assimilate transport?" in *Hormone Action in Plant Development–A Critical Appraisal*, eds G. V. Hoad, M. B. Jackson, and J. R. Lenton (Butterworths), 175–187.

Patrick, J. W. (1990). Sieve element unloading: cellular pathway, mechanism and control. *Physiol. Plant.* 78, 298–308. doi: 10.1111/j.1399–3054.1990.tb02095.x

Patrick, J. W., and Turvey, P. M. (1981). The pathway of radial transfer of photosynthate in decapitated stems of *Phaseolus vulgaris* L. *Ann. Bot.* 47, 611–621.

Patrick, J. W. (2012). "Fundamentals of phloem transport physiology," in *Phloem: Molecular Cell Biology, Systemic Communication, Biotic Interactions*, eds G. A. Thompson and A. J. E. van Bel (London: Wiley-Blackwell Publishing), 30–60.

Patrick, J. W., and Offler, C. E. (1996). Post-sieve element transport of photoassimilates in sink regions. *J. Exp. Bot.* 47, 1165–1178. doi: 10.1093/jxb/47.Special_Issue.1165

Pinkard, E. A., Eyles, A., and O'Grady, A. P. (2011). Are gas exchange responses to resource limitation and defoliation linked to source:sink relationships? *Plant Cell Environ.* 34, 16552–11665. doi: 10.1111/j.1365–3040.2011.02361.x

Prat, S. (2010). "Hormonal and daylength control of potato tuberization," in *Plant Hormones. Biosynthesis, Signal Transduction, Action!* ed. P. J., Davies (Springer), 574–596.

Prichard, J. (1996). Aphid stylectomy reveals an osmotic step between sieve tube and cortical cells in barley roots. *J. Exp. Bot.* 47, 1519–1524. doi: 10.1093/jxb/47.10.1519

Pritchard, J., Winch, S., and Gould, N. (2000). Phloem water relations and root growth. *Aust. J. Plant Physiol.* 27, 539–548. doi: 10.1071/PP99175

Pritchard, J., Tomos, A. D., Farrar, J. F., Minchin, P. E. H., Gould, N., Paul, M. J., et al. (2004). Turgor, solute import and growth in maize roots treated with galactose. *Funct. Plant Biol.* 31, 1095–1103. doi: 10.1071/FP04082

Pritchard, J., Ford-Llyod, B., and Newbury, H. J. (2005). "Roots as an integrated part of the translocation pathway," in *Vascular Transport in Plants*, eds N. M. Holbrook and M. Zwieniecki (Amsterdam: Elsevier), 157–180.

Proseus, T. E., and Boyer, J. S. (2005). Turgor pressure moves polysaccharides into growing cell walls of *Chara corallina*. *Ann. Bot.* 95, 967–979. doi: 10.1093/aob/mci113

Qian, T., Dieleman, J. A., Eling, A., and Marcelis, L. F. M. (2012). Leaf photosynthetic and morphological responses to elevated CO_2 concentration and altered fruit number in the semi-closed greenhouse. *Sci. Hort.* 145, 1–9. doi: 10.1016/j.scienta.2012.07.015

Radchuk, V., Radchuk, R., Pirko, Y., Vankova, R., Gaudinova, A., Korkhovy, V., et al. (2012). A somaclonal line SE7 of finger millet (*Eleusina coracea*) exhibits modified cytokinin homeostasis and increased grain yield. *J. Exp. Bot.* 63, 5497–5506. doi: 10.1093/jxb/ers200

Rosche, E., Blackmore, D., Tegeder, M., Richardson, T., Schroeder, H., Higgins, T. J. V., et al. (2002). Seed-specific expression of a potato sucrose transporter increases sucrose uptake and growth rates of developing pea cotyledons. *Plant J.* 30, 165–175. doi: 10.1046/j.1365-313X.2002.01282.x

Ruan, Y. L., Patrick, J. W., Bouzayen, M., Osario, S., Fernie, A. R. (2012). Molecular regulation of seed and fruit development. *TIPS* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Rutschow, H. L., Baskin, T. I., and Kramer, E. M. (2011). Regulation of solute flux through plasmodesmata in the root meristem. *Plant Physiol.* 155, 1817–1826. doi: 10.1104/pp.110.168187

Sala, A., Woodruff, D. R., and Meinzer, F. C. (2012). Carbon dynamics in trees: feast or famine? *Tree Physiol.* 32, 764–775. doi: 10.1093/treephys/tpr143

Saladié, M., Matas, A. J., Isaacson, T., Jenks, M. A., Goodwin, S. M., Niklas, K. J., et al. (2007). A reevaluation of the key factors that influence tomato fruit softening and integrity. *Plant Physiol.* 144, 1012–1028. doi: 10.1104/pp.107.097477

Scofield, G. N., Hirose, T., Aoki, N., and Furbank, R. T. (2007). Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. *J. Exp. Bot.* 58, 3155–3169. doi: 10.1093/jxb/erm153

Schulz, A. (1994). Phloem transport and differential unloading in pea seedlings after source and sink manipulations. *Planta* 192, 239–248. doi: 10.1007/BF01089040

Schulz, A. (1995). Plasmodesmal widening accompanies the short-term increase in symplasmic phloem unloading in pea root tips under osmotic stress. *Protoplasma* 188, 22–37. doi: 10.1007/BF01276793

Smidansky, E. D., Clancy, M., Meyer, F. D., Lanning, S. P., Blake, N. K., Talbert, L. E., et al. (2002). Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increasaes seed yield. *PNAS* 99, 1724–1729. doi: 10.1073/pnas.022635299

Smidansky, E. D., Martin, J. M., Hannah, C., Fischer, A. M., and Giroux, M. J. (2003). Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. *Planta* 217, 656–664. doi: 10.1007/s00425-002-0897-z

Smidansky, E. D., Meyer, F. D., Blakeslee, B., Weglaz, T. E., Greene, T. W., and Giroux, M. J. (2007). Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism. *Planta* 225, 965–976. doi: 10.1007/s00425-006-0400–3

Smith, J. A. C., and Milburn, J. A. (1980a). Osmoregulation and the control of phloem-sap composition in *Ricinus communis*. *Planta* 148, 28–34.

Smith, J. A. C., and Milburn, J. A. (1980b). Phloem turgor and the regulation of sucrose loading in Ricinus communis. *Planta* 148, 42–48.

Sovonick-Dunford, S., Lee, D. R., and Zimmermann, M. H. (1981). Direct and indirect measurements of phloem turgor pressure in white ash. *Plant Physiol.* 68, 121–126.

Stadler, R., Wright, K. M., Lauterbach, C., Amon, G., Gahrtz, M., Feuerstein, A., et al. (2005a). Expression of GFP-fusions in Arabidopsis companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. *Plant J.* 41, 319–331. doi: 10.1111/j.1365-313X.2004.02298.x

Stadler, R., Lauterbach, C., and Sauer, N. (2005b). Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in Arabidopsis seeds and embryos. *Plant Physiol.* 139, 701–712. doi: 10.1104/pp.105.065607

Tegeder, M., Ruan, Y.-L., and Patrick, J. W. (2012). "Roles of plasma membrane transporters in phloem functions," in *Phloem Molecular Cell Biology, Systemic Communication, Biotic Interactions,* eds G. A. Thompson and A. E. J. van Bel (Hoboken: John Wiley and Sons, Inc.), 63–101. doi: 10.1002/9781118382806.ch4

Thomas, M., Hetherington, L., and Patrick, J. W. (2000). Genotypic differences in seed growth rates of *Phaseolus vulgaris* L. General characteristics, seed coat factors and comparative roles of seed coats and cotyledons. *Aust. J. Plant Physiol.* 27, 109–118. doi: 10.1071/PP99116

Thompson, M. V. (2006). Phloem: the long and the short of it. *Trends Plant Sci.* 11, 26–32. doi: 10.1016/j.tplants.2005.11.009

Thompson, M. V., and Holbrook, N. M. (2003). Scaling phloem transport: water potential equilibrium and osmoregulatory flow. *Plant Cell Environ.* 26, 1561–1577. doi: 10.1046/j.1365-3040.2003.01080.x

Thorpe, M., Minchin, P., Gould, N., and McQueen, J. (2005). "The stem apoplast: a potential communication channel in plant growth regulation," in *Vascular Transport in Plants*, eds N. M. Holbrook and M. A. Zwieniecki (Amsterdam: Elsevier Academic Press), 201–220.

Turgeon, R. (2010). The puzzle of phloem pressure. *Plant Physiol.* 154, 578–581. doi: 10.1104/pp.110.161679

Turvey, P. M., and Patrick, J. W. (1979). Kinetin-promoted transport of assimilate in stems of *Phaseolus vulgaris* L. Localised versus remote site(s) of action. *Planta* 147, 151–155. doi: 10.1007/BF00389516

van Bel, A. J. E., and van Rijen, H. V. M. (1994). Microelectrode-recorded development of the symplasmic autonomy of the sieve element/companion cell complex in the stem phloem of Lupinus luteus L. *Planta* 192, 165–175. doi: 10.1007/BF01089031

van Bel, A. J. E., and Hafke, J. B. (2005). "Physicochemical determinants of phloem transport," in *Vascular Transport in Plants*, eds N. M. Holbrook and M. A. Zwieniecki (Amsterdam: Elsevier Academic Press), 19–44.

Viola, R., Roberts, A. G., Haupt, S., Gazzani, S., Hancock, R. D., Marmiroli, N., et al. (2001). Tuberization in potato involves a switch from apoplastic to symplastic phloem unloading. *Plant Cell* 13, 385–398. doi: 10.1105/tpc.13.2.385

Voitsekhovskaja, O. V., Rudashevskaya, E. L., Demchenko, K. N., Pakhomova, M. V., Batashev, D. R., Gamelei, Y. V., et al. (2009). Evidence for functional heterogeneity of sieve element-companion cell complexes in minor veins phloem of Alonsoa meridionalis. *J. Exp. Bot.* 60, 1873–1883. doi: 10.1093/jxb/erp074

Wada, H., Matthews, M. A., Shakel, K. A. (2009). Seasonal pattern of apoplastic solute accumulation and loss of cell turgor during ripening of *Vitis vinifera* fruit under field conditions. *J Exp. Bot.* 60, 1773–1781. doi: 10.1093/jxb/erp050

Wang, H. L., Offler, C. E., and Patrick, J. W. (1995). Cellular pathway of photosynthate transfer in the developing wheat grain. II. A structural analysis and histochemical studies of the transfer pathway from the crease phloem to the endosperm cavity. *Plant Cell Environ.* 18, 373–388. doi: 10.1111/j.1365-3040.1995.tb00373.x

Wardlaw, I. F. (1990). The control of carbon partitioning in plants. *New Phytol.* 116, 341–381. doi: 10.1007/PL00008146

Wardlaw, I. F., and Moncur, L. (1976). Source, sink and hormonal-control of translocatioin in wheat. *Planta* 128, 93–100. doi: 10.1007/BF00390309

Warmbrodt, R. D. (1987). Solute concentrations in the phloem and apex of the root of Zea mays. *Am. J. Bot.* 74, 394–402.

Watson, B. T. (1976). Rapid propogation of changes in velocity of translocation along the phloem pathway of *Helianthus annuus* L. *Ann. Bot.* 40, 659–667.

Weichert, N., Saalbach, I., Weichert, H., Kohl, S., Erban, A., Kopka, J., et al. (2010). Increasing sucrose uptake capacity of wheat grains stimulates storage protein synthesis. *Plant Physiol.* 152, 698–710. doi: 10.1104/pp.109.150854

Werner, D., Gerlitz, N., and Stadler, R. (2011). A dual switch in phloem unloading during ovule development in Arabidopsis. *Protoplasma* 248, 225–235. doi: 10.1007/s00709-010-0223-8

White, R. G., and Barton, D. A. (2011). The cytoskeleton in plasmodesmata: a role in intercellular transport? *J. Exp. Bot.* 62, 5249–5266. doi: 10.1093/jxb/err227

Winch, S., and Pritchard, J. (1998). Acid-induced wall loosening is confined to the accelerating region of the root growing zone. *J. Exp. Bot.* 50, 1481–1487.

Windt, C. W., Vergeldt, F. J., de Jager, P. A., and Van As, H. (2006). MRI of long-distance water transport: a comparison of the phloem and xylem flow characteristics and dynamics in poplar, castor bean, tomato and tobacco. *Plant Cell Environ.* 29, 1715–1729. doi: 10.1111/j.1365-3040.2006.01544.x

Wright, J. P., and Fisher, D. R. (1980). Direct measurement of sieve tube turgor pressure using severed aphid stylets. *Plant Physiol.* 65, 1133–1135. doi: 10.1104/pp.65.6.1133

Wright, J. P., and Fisher, D. B. (1983). Estimation of the volumetric elastic modulus and membrane hydraulic conductivity of willow sieve tubes. *Plant Physiol.* 73, 1042–1047. doi: 10.1104/pp.73.4.1042

Wu, L., and Birch, R. G. (2007). Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotech.* 5, 109–117. doi: 10.1111/j.1467-7652.2006.00224.x

Wu, G. L., Zhang, X. Y., Zhang, L. Y., Pan, Q. H., Shen, Y. Y., and Zhang, D. P. (2004). Phloem unloading in developing walnut fruit is symplasmic in the seed pericarp and apoplasmic in the fleshy pericarp. *Plant Cell Physiol.* 45, 1461–1470. doi: 10.1093/pcp/pch169

Xu, M., Cho, E., Burch-Smith, T. M., and Zambryski, P. C. (2012). Plasmodesmata formation and cell-to-cell transport are reduced in decreased size exclusion limit 1 during embryogenesis in Arabidopsis. *PNAS* 109, 5098–5103. doi: 10.1073/pnas.1202919109

Zhang, L.-Y., Peng, Y. B., Pelleschi-Travier, S., Fan, Y., Lu, Y. F., Lu, Y. M., et al. (2004). Evidence for apoplasmic phloem unloading in developing apple fruit. *Plant Physiol.* 135, 1–13. doi: 10.1104/pp.103.036632

Zhang, X.-Y., Wang, X.-L., Wang, X.-F., Xia, G.-H., Pan, Q.-H., Fan, R.-C., et al. (2006). A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. *Plant Physiol.* 142, 220–232. doi: 10.1104/pp.106.081430

Zhang, W.-H., Zhou, Y., Dibley, K. E., Tyerman, S. D., Furbank, R. T., and Patrick, J. W. (2007a). Nutrient loading of developing seeds. *Funct. Plant Biol.* 34, 314–331. doi: 10.1071/FP06271

Zhang, W.-H., Patrick, J. W., and Tyerman, S. D. (2007b). Actin filaments modulate hypoosmotic-responsive K+ efflux channels in specialised cells of developing bean seed coats. *Funct. Plant Biol.* 34, 874–884. doi: 10.1071/FP07138

Zhu, T., Lucas, W. J., and Rost, T. L. (1998). Directional cell-to-cell communication in the Arabidopsis root apical meristem. I. An ultrastructural and functional analysis. *Protoplasma* 203, 35–47. doi: 10.1007/BF01280585

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Regulation of assimilate import into sink organs: update on molecular drivers of sink strength

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Recent developments have altered our view of molecular mechanisms that determine sink strength, defined here as the capacity of non-photosynthetic structures to compete for import of photoassimilates. We review new findings from diverse systems, including stems, seeds, flowers, and fruits. An important advance has been the identification of new transporters and facilitators with major roles in the accumulation and equilibration of sugars at a cellular level. Exactly where each exerts its effect varies among systems. Sugarcane and sweet sorghum stems, for example, both accumulate high levels of sucrose, but may do so via different paths. The distinction is central to strategies for targeted manipulation of sink strength using transporter genes, and shows the importance of system-specific analyses. Another major advance has been the identification of deep hypoxia as a feature of normal grain development. This means that molecular drivers of sink strength in endosperm operate in very low oxygen levels, and under metabolic conditions quite different than previously assumed. Successful enhancement of sink strength has nonetheless been achieved in grains by up-regulating genes for starch biosynthesis. Additionally, our understanding of sink strength is enhanced by awareness of the dual roles played by invertases (INVs), not only in sucrose metabolism, but also in production of the hexose sugar signals that regulate cell cycle and cell division programs. These contributions of INV to cell expansion and division prove to be vital for establishment of young sinks ranging from flowers to fruit. Since INV genes are themselves sugar-responsive "feast genes," they can mediate a feed-forward enhancement of sink strength when assimilates are abundant. Greater overall productivity and yield have thus been attained in key instances, indicating that even broader enhancements may be achievable as we discover the detailed molecular mechanisms that drive sink strength in diverse systems.

Keywords: carbohydrate partitioning, kernel, maize, sink strength, sorghum, stem, sucrose, sugarcane

INTRODUCTION

Whole-plant carbohydrate partitioning is the process by which the products of atmospheric carbon dioxide assimilation are exported from leaves through the veins to distant non-photosynthetic tissues. Researchers aiming to improve crop yield have focused on two key aspects of whole-plant carbohydrate partitioning: enhancing carbohydrate production in leaves (i.e., increasing source capacity) and/or improving the utilization of photoassimilates by sink organs (i.e., enhancing sink strength). Examples of strategies to enhance carbohydrate production include attempts to increase light interception either by increasing the number of leaves or total leaf area, breeding for stay-green traits, and enhancing photosynthesis by improving the capacity of the plant to fix carbon (Sakamoto et al., 2006; Hammer et al., 2009; Zheng et al., 2009; Zhu et al., 2010; Raines, 2011; Ruan et al., 2012a). In regards to the strategy of enhancing sink strength, researchers are attempting to increase the number, size, and carbohydrate-storing activity of sink organs (Ho, 1988; Marcelis, 1996; Herbers and Sonnewald, 1998; Smidansky et al., 2002). Sink strength has been defined as the competitive ability of a sink organ to import photoassimilates, and depends on both its physical (size) and physiological (activity) capabilities (Ho, 1988; Marcelis, 1996; Herbers and Sonnewald, 1998).

Carbon fixation occurs in source leaves and is biochemically driven by photosynthesis. The fixed carbon, primarily in the form of sucrose, moves from mesophyll cells into the phloem, where it is transported long-distance through veins into sink tissues. Carbohydrates assimilated during the day in excess of leaf export capacity are transiently stored as starch in chloroplasts and as sucrose in the vacuole, and then remobilized during the night to continuously supply sink tissues (Rhoades and Carvalho, 1944; Kaiser and Heber, 1984; Smith and Stitt, 2007; Slewinski et al., 2008; Eom et al., 2011). The imported carbon is then either directed into immediate usage in metabolic processes important for growth and development or stored inside sink organs (Gifford and Evans, 1981). In source leaves, assimilated carbon can

move into the phloem entirely through a symplastic (cytoplasmic) path, or it may first be exported to the apoplast (cell wall space) surrounding the phloem, prior to being imported into phloem companion cells and/or sieve elements (Lalonde et al., 2004; Braun and Slewinski, 2009). In the symplastic pathway, transport of sugar occurs cell-to-cell through plasmodesmatal connections, passively flowing down a concentration gradient. In contrast, in the apoplastic path, the distribution of fixed carbon can move against a concentration gradient in an energy-dependent process. In this case, sugar transporters import sugars from the phloem apoplast across the cell membrane, resulting in its accumulation to high levels in the cytoplasm (Turgeon, 2006; Ayre, 2011). Consumption and storage of sugar can be controlled at various points along the transport path. These regulatory points include phloem unloading from vascular tissues into storage parenchyma cells, compartmentation at the tonoplast of storage parenchyma cells, and metabolism and/or storage within sink tissues (Patrick, 1997). Additionally, diverse environmental factors can also affect sink strength (Marcelis, 1996; Andersen, 2003; Aranjuelo et al., 2011). In this review, we discuss recent findings related to the molecular drivers of sink strength in stems, seeds, flowers, and fruits. For recent progress discussing source strength and phloem loading in leaves, see Lalonde et al. (2004), Turgeon (2006), Sauer (2007), Ma et al. (2008), Braun and Slewinski (2009), Kühn and Grof (2010), Slewinski and Braun (2010), Ainsworth and Bush (2011), Ayre (2011), Baker et al. (2012), Braun (2012), and Chen et al. (2012).

THE STEM AS A STORAGE ORGAN

In plants, storage organs are structures that accumulate carbohydrates derived from photosynthesis. They include fruits, seeds, roots, and/or stems. An example of stem acting as a storage organ is the potato (*Solanum tuberosum*) tuber, which is a modified stem capable of storing large amounts of starch (Wiltshire and Cobb, 1996). Other examples are found in grasses, such as sweet sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*; Slewinski, 2012). As members of the Panicoideae subfamily, both plants are C_4 species that can accumulate high concentrations of fermentable sugars, mainly sucrose, in their stems. Because of the high stem sucrose contents, both plants are models for strong stem sinks, and are being used for the production of ethanol as a biofuel (Waclawovsky et al., 2010; Calviño and Messing, 2012). These plants will be the focus of the first part of the review.

In both sweet sorghum and sugarcane, photoassimilates are first used for plant growth and development during early vegetative stages. Afterward, when the internodes (the sections of the stem between two nodes) have elongated, stems transition to sugar storage organs, where most of the accumulated carbon is stored as sucrose (Hoffmann-Thoma et al., 1996; Lingle, 1999; Almodares et al., 2008; Almodares and Hadi, 2009; Calviño and Messing, 2012; Slewinski, 2012). It has been proposed that stored sugars in the stem are used to buffer photoassimilate supply to the grains during plant growth and development. Both sweet sorghum and sugarcane stems exhibit a developmentally progressive increase in sucrose content in which they accumulate higher concentrations of sucrose in more mature internodes (i.e., lower down the stem) compared to younger ones (i.e., upper; Batta and Singh, 1986; Hoffmann-Thoma et al., 1996; McCormick et al., 2006; Tarpley and Vietor, 2007; Slewinski, 2012).

Even though sweet sorghum and sugarcane are closely related and both accumulate large amounts of sucrose in their stems, there are similarities and differences in regards to their sucrose accumulation. In both cases, there is a developmental decrease in the activity of sucrose metabolizing enzymes, particularly acid invertases (INV) as stem elongation nears completion (Ruan et al., 2010). Later, however, the radial transfer of sucrose from the phloem into the storage cells of the mature internodes may follow a different path in these two grasses (see below). Evidence indicates the presence of distinctive mechanisms and the possible involvement of different molecular and physiological drivers underpinning the accumulation of high levels of sucrose in sweet sorghum and sugarcane stems.

Sweet sorghum cultivars brought to the United States, mainly from China and Africa, were subject to selection and improvement for disease resistance, high stem sugar content, stalk erectness, and biomass accumulation (Reddy et al., 2005; Rooney et al., 2007). Because of this selection-based bottleneck effect, little variation is found within cultivated sweet sorghum varieties, especially in terms of stem sugar composition (Ali et al., 2008; Murray et al., 2009). Likewise, since the major focus in sugarcane improvement programs has been to increase stem sugar content, the genetic variation related to this trait in germplasm collections used in breeding programs is also limited (Aitken et al., 2008; Creste et al., 2010; Mancini et al., 2012). However, despite this narrow genetic variation in the sweet sorghum and sugarcane germplasms, the presence of subgroups with different sugar contents has been reported in each species. Two groups of sweet sorghum have been identified using simple sequence repeats (SSRs) markers: group IX with high sugar content and group VII with low sugar yield (Ali et al., 2008). Additionally, Murray et al. (2009) used both SSR and single nucleotide polymorphism (SNP) markers to genotype 125 cultivars of sorghum, and classified the sweet sorghum varieties into three major groups based on differing stem sugar contents. Similarly, using amplified fragment length polymorphism (AFLP) and SSR markers, two types of sweet sorghum with different brix content (a measure of the level of solutes in stem juice) were detected in a germplasm collection in Mexico (Pecina-Quintero et al., 2012). Likewise, differences were observed in sugar composition and content from eight sugarcane varieties, including four commercial cultivars (Tai and Miller, 2002). These findings suggest that genetic differences exist in the control of sink strength within each species, which should be considered when investigating the molecular drivers of sugar accumulation.

SUGAR TRANSPORT AND UNLOADING INTO STEM PARENCHYMA CELLS

Transport path

Sugarcane and sweet sorghum may differ in their strategies for accumulating high levels of sucrose in their stems. In maturing sugarcane stems, sucrose transport follows a predominantly symplastic pathway when moving from the phloem into the storage parenchyma cells (**Figure 1A**; Lingle, 1989; Moore and Cosgrove, 1991; Rae et al., 2005; Patrick et al., 2013). This is supported by evidence that the sap collected from the xylem of sugarcane stems,

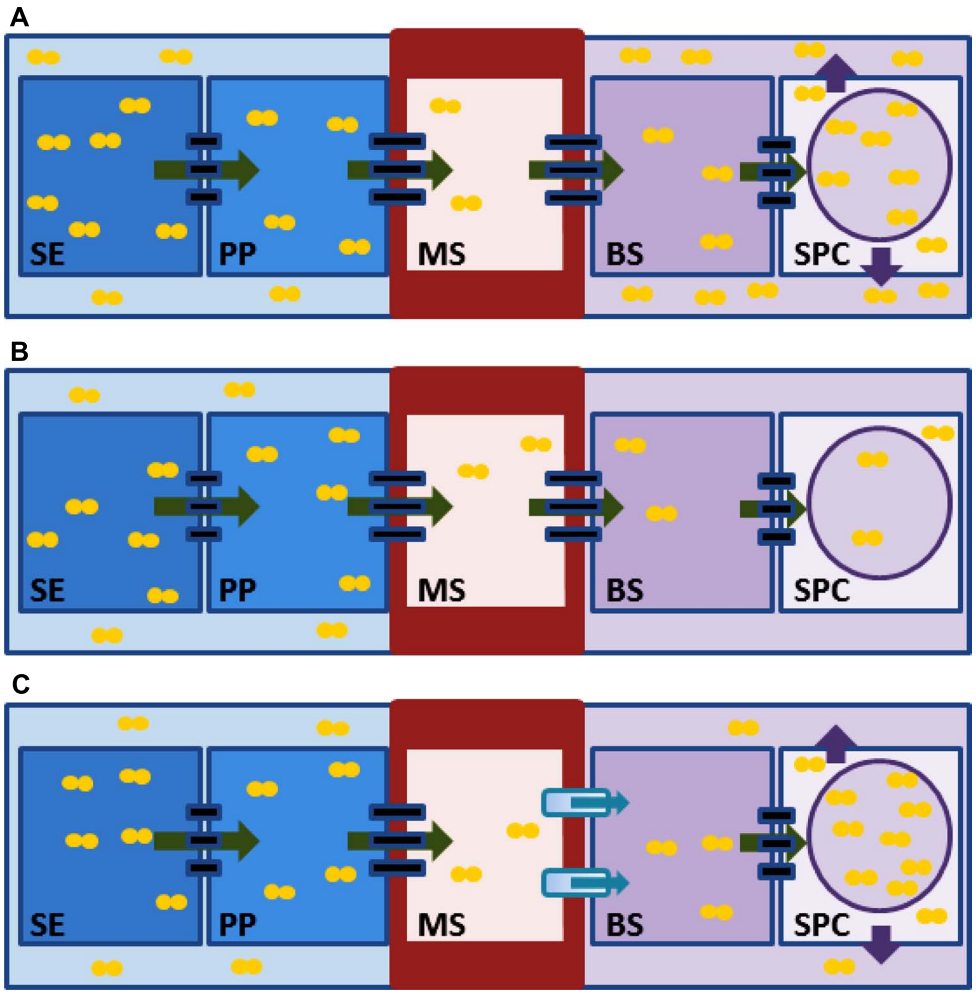

FIGURE 1 | Models for sucrose movement in sugarcane and sweet sorghum stems. (A) In mature sugarcane stems, sucrose (orange circles) moves symplastically cell-to-cell (green arrows) through plasmodesmata (black rectangles) from the phloem sieve element (SE) to the stem parenchyma cell (SPC). In the SPC, some of the sucrose is stored in the vacuole (purple circle), and some is released to the apoplast (purple arrows) to increase the sink strength and sucrose storage capacity of the tissue. Sucrose in the SPC apoplast is prevented from diffusing back into the phloem apoplast by the suberized and lignified cell wall (thick red outline) of the mestome sheath cells (MS). **(B)** In immature sorghum stems, sucrose follows a symplastic path from the SE to the SPC cells. This tissue is actively growing and not storing much sucrose. **(C)** In ripening sorghum stems, sucrose movement from the SE to the SPC includes an apoplastic step. The suberized and lignified cell wall of the MS cell necessitates that the sucrose efflux must occur from either the MS or the bundle sheath cell (BS, not shown). Import could occur in either the BS or in the SPC (not shown). For simplicity, sucrose efflux and import are shown at the MS–BS interface by the shaded blue rectangle and blue arrow. The majority of the stored sucrose is intracellular. PP, phloem parenchyma cell.

which is contiguous with the phloem apoplast, contains virtually no sucrose (Welbaum et al., 1992), and by the observation that a fluorescent dye loaded into leaves is unloaded from the stem phloem and able to move symplastically through plasmodesmata to the stem parenchyma cells (Rae et al., 2005). In the stem, as sucrose begins to accumulate, some of it is effluxed to the apoplast of stem parenchyma cells by turgor-driven homeostatic leakage, and the apoplastic sucrose concentration can reach approximately 400–700 mM (Welbaum and Meinzer, 1990; Moore and Cosgrove, 1991; Welbaum et al., 1992; Patrick et al., 2013). Sucrose accumulation in the stem parenchyma cell apoplast serves as an additional storage compartment and increases sink strength. At maturity, when sucrose accumulates to very high levels in the stem apoplast, sucrose backflow into the phloem is prevented by the lignified and suberized cell walls of the inner, mestome sheath (a ring of thick-walled cells that surrounds the vein internally to the bundle sheath cells; Welbaum et al., 1992; Rae et al., 2005; Walsh et al., 2005; Patrick et al., 2013). This structural adaptation is also present in sorghum (Artschwager, 1948) and presumably functions to isolate the parenchyma cell apoplast from the phloem apoplast.

Sucrose movement in sorghum initially follows a symplastic path into the growing internode (**Figure 1B**), but is reported to later include an apoplastic path during internode ripening (**Figure 1C**; Tarpley et al., 1996; Tarpley and Vietor, 2007). This model is based upon the specific activity of stem-infused radiolabeled sucrose recovered from growing axillary branches vs.

ripening internodes. Additional support for this scenario using alternative approaches, such as determining whether the sucrose transporters implicated in apoplastic transport (see below) are required to import sucrose across the plasma membrane of the stem parenchyma cells, is needed. Approximately 80–90% of the sugar in sorghum stem internodes accumulates intracellularly within the parenchyma cells, and only 10–20% of the sugar accumulates in the stem apoplast (Tarpley and Vietor, 2007). The five- to ten-fold difference in sucrose levels between the symplast and apoplast of stem parenchyma cells suggests that the mechanisms of sorghum and sugarcane sucrose storage may be distinct. However, two caveats to this model need to be considered. First, the differences reported in intracellular vs. apoplastic sucrose concentrations were measured using two grain sorghum lines rather than sweet sorghum materials, and therefore, genetic differences between these sorghum types could explain the observed differences from sugarcane. Second, it is possible that sorghum uses a similar mechanism of turgor-driven sucrose leakage to the apoplast in mature stem parenchyma cells. In their study, Tarpley and Vietor (2007) used ripening internodes for their measurements of sucrose compartmentation, which could reflect that the storage cells had not reached their maximal capacity and not yet effluxed sucrose to its final concentration in the apoplast. In support of this hypothesis, a previous analyses of sugar content using the same stem-infusion technique in two other sorghum varieties, comparing plants at the grain filling stage to those 2 weeks after grain maturity, showed that sucrose levels continued to increase in the apoplast free space compared to the intracellular compartment (Tarpley et al., 1996). Hence, the symplastic and apoplastic sucrose contents in sorghum stems at final maturity may not be as different from what was found for sugarcane. Further work is necessary to characterize the transport path and compartmentation of sucrose in sweet sorghum and to determine if it utilizes a different mechanism than sugarcane.

Sugar transporters

In sweet sorghum, biochemical studies have shown that sucrose must cross the plasma membrane and tonoplast for storage in the stem parenchyma cells, indicating the involvement of sugar transporters (Hoffmann-Thoma et al., 1996). Sucrose transporters (SUTs) are H^+/sucrose symporters and are the most-studied class of transporters capable of translocating sucrose across a membrane (Lalonde et al., 2004; Sauer, 2007; Braun and Slewinski, 2009; Kühn and Grof, 2010; Ainsworth and Bush, 2011; Ayre, 2011). In the sorghum genome, there are six SUTs (SUT1–6) that have been proposed to encode proteins located in the plasma membrane and/or tonoplast (Braun and Slewinski, 2009).

The hypothesis that transporters are involved in high sucrose accumulation was recently examined in sweet sorghum. Transcriptional analyses revealed that SUT mRNAs, specifically SUT1 and SUT4, correlate with high sugar content in sweet sorghum stems when compared to grain sorghum stems (Qazi et al., 2012). Still, the exact roles of SUTs in sucrose accumulation in stem sink tissues remain undefined. In maize, SUT1 was found to be expressed in stems (Aoki et al., 1999); however, its function in stem tissues is unknown as it has only been shown to function in sucrose loading into the phloem in leaves (Slewinski et al., 2009, 2010). In sugarcane, using a peptide antibody raised against the orthologous SUT1 protein (ShSUT1), Rae et al. (2005) found that ShSUT1 was expressed in the mestome sheath and vascular parenchyma cells in stem and leaf tissues, suggesting SUT1 has non-phloem-loading functions in this species. The authors proposed a role for ShSUT1 in retrieving sucrose from the apoplast as a biochemical barrier to maintain the sucrose gradient between the phloem and stem storage parenchyma cells. Thus, it is possible that the SUT1 RNA expression detected in sweet sorghum stems (Qazi et al., 2012) reflects a similar role to the closely related ShSUT1. More work is needed to determine the function of SUT genes in stem sucrose partitioning.

Recently, other sugar transporter proteins, such as SWEETs and tonoplast monosaccharide transporters (TMTs), were shown to transport sucrose across membranes (Wingenter et al., 2010; Chen et al., 2012). However, the roles of these additional sucrose transporters in facilitating sucrose transfer into the stem have not been characterized (Ayre, 2011; Slewinski, 2011, 2012; Baker et al., 2012; Braun, 2012; Chen et al., 2012).

SUGAR METABOLISM AND STORAGE IN THE STEM

Sucrose metabolism and storage processes in sink cells represent an important determinant of stem sink strength. During initial expansion of sweet sorghum and sugarcane stems, INVs contribute to sink strength and stem size. However, later, sucrose is stored in the large vacuoles of stem parenchyma cells. Very little of this stored sucrose is hydrolyzed during its transfer into the ripening stem (Lingle, 1989; Tarpley et al., 1996; Tarpley and Vietor, 2007). Further, after the stems have matured and elongation has ceased, these tissues show low metabolic activity (Tarpley et al., 1996), prompting the question as to whether sucrose metabolism remains an important driver of sink strength in maturing sweet sorghum and sugarcane stem tissues.

Several key enzymes involved in sucrose metabolism are INV, sucrose synthase (SUS), sucrose phosphate synthase (SPS), and sucrose phosphate phosphatase (SPP). INV enzymes catalyze the cleavage of sucrose into glucose and fructose, and various types of INV enzymes are found in plants and function in different locations, including the cell wall, vacuole, and the cytoplasm (Ruan et al., 2010; Vargas and Salerno, 2010; Patrick et al., 2013). SUS is another enzyme that cleaves sucrose into fructose and UDP-glucose in a reversible reaction (Winter and Huber, 2000; Patrick et al., 2013). Finally, SPS and SPP play an important role in sucrose synthesis in the cell. In the cytosol, SPS and SPP are jointly responsible for the irreversible synthesis of sucrose from UDP-glucose and fructose-6-phosphate (Lunn and MacRae, 2003).

Compared to sweet sorghum, grain sorghum accumulates little sucrose in the stem, and most of the carbohydrates produced are directed toward storage in seeds (Tarpley et al., 1994; Calviño and Messing, 2012; Qazi et al., 2012). This contrast suggests that sink strength differs in the seeds and stems between the two varieties. By examining sugar accumulation and storage in three cultivars of sweet sorghum, Hoffmann-Thoma et al. (1996) ascribed negligible roles for the three key sucrose metabolizing enzymes SUS, SPS, and INV. Similarly, Qazi et al. (2012) compared sugar accumulation between a sweet and a grain sorghum variety at three developmental stages in both the upper and lower internodes.

They found that variety, stage, and internode position contributed to differences seen in sugar content. However, differences were not detected in the activities of SUS, SPS, and INV between the two varieties, indicating these enzymes are not likely to be primary contributors to the variation observed in stem sugar content.

In the sugarcane stem, sucrose accumulation has been linked to the differential RNA or protein expression or localization of enzymes involved in sucrose metabolism (Schafer et al., 2004; Grof et al., 2006). However, because of the complexities of stem tissues being composed of multiple cell types and the developmental progression in stem sucrose accumulation, correlations between expression and function may not always be evident. Nonetheless, in some cases, the activities of sucrose metabolizing enzymes are thought to regulate sucrose levels in sink tissues (Zhu et al., 1997; Botha and Black, 2000; Koch, 2004; Grof et al., 2007). For instance, soluble acid INV activity is low during maturation in varieties that store high levels of sugar (Zhu et al., 1997). Meanwhile, at least three SUS isoforms are present in sugarcane, and sucrose synthesis is correlated with SUS activity. In addition, the ratio of sucrose synthesis to breakdown is higher in mature internodes, and researchers have suggested that different isoforms of SUS are expressed in young internodes compared to mature ones (Schafer et al., 2004). A positive relationship between SPS activity and sucrose accumulation has also been demonstrated in sugarcane (Botha and Black, 2000; Grof et al., 2007). However, studies using enzyme activity and isotope tracers have demonstrated that sucrose can be transferred from phloem tissues into the vacuoles of storage parenchyma cells without being catabolized and then resynthesized in the stem (Lingle, 1989). Collectively, these data suggest that the enzymes involved in sucrose metabolism likely play a minor role in sucrose storage by the maturing stem and are unlikely to be major drivers of stem sink strength at later stages of development in sugarcane.

It thus appears that additional processes besides sucrose metabolizing enzymes may be important contributors to the high level of sucrose accumulation in sugarcane stems. In the case of sorghum, where quantitative trait loci (QTL) mapping is possible, researchers have identified genomic regions related to sucrose accumulation in the stem (Murray et al., 2008a,b; Ritter et al., 2008). However, the genes underlying these QTLs have yet to be identified. Transcriptional profiling experiments to characterize gene expression changes between stem tissues of sweet and grain sorghum have also been conducted (Calviño et al., 2009). The researchers identified several intriguing candidate genes that correlate with high sucrose content in sweet sorghum, but follow up studies to demonstrate causality have not been reported. The identification of genes that positively regulate sucrose content in grass stems is a high priority for biotechnological approaches to improving biomass feedstocks for biofuels production.

APPROACHES TOWARD INCREASING STEM SINK STRENGTH

Because of the importance of sucrose stored in the stem for both food and biofuel production, increasing its concentration in stem storage parenchyma cells is a major research objective. There are a number of avenues that researchers could target in order to improve stem sink strength. In addition to selecting sweeter varieties (reflecting higher content of sugar in the stem), one approach is to increase stem size, which would mirror an increase in cell number and/or an increase in individual cell expansion. Moreover, there is the potential to increase surface/unloading area for sugars into stem storage parenchyma cells. This is the theory behind breeding efforts that have targeted and successfully led to thicker stems, higher stem juice volume, and increased sucrose concentration in the internodes of both sugarcane and sweet sorghum (Glassop et al., 2007; Slewinski, 2012; Patrick et al., 2013).

Another approach to increasing sucrose content in stems is by reducing the sink strength of other organs (e.g., grains) and thereby reducing competition for photoassimilates. This approach could be significant because competition among sink organs is known to be higher when supply is limited (Marcelis, 1996; Andersen, 2003). Breeding efforts have led to the generation of sweet sorghum varieties with larger stems and reduced panicle size. However, other sorghum lines have been reported to have high stem sugar concentration and still maintain seed yields comparable to those of grain sorghum (Qazi et al., 2012). At the opposite extreme, enhanced sink strength was engineered by removing all but one of the source leaves from sugarcane plants. This manipulation of the sink/source ratio in sugarcane provided evidence for source limitation under high sink demand (McCormick et al., 2006). These data also demonstrated active communication and influence on source activity by sinks tissues, namely, photosynthetic rate increased in the one remaining source leaf to provide sufficient photoassimilates to meet sink demands. Hence, supply and demand equilibrium is important to overall productivity; therefore, targeting one aspect alone might not lead to increased sugar content (McCormick et al., 2009). Thus, there is a great need to study the control of carbon flux in stems, the functions of the genes responsible, and also to determine the limiting factors/enzymes that can be targeted for manipulation in order to achieve the goal of enhancing endogenous sugar content in stem tissues.

Along these lines, caution is warranted when using a transgenic approach to improve stem sugar content by targeting a sucrose metabolizing enzyme, since these efforts have not yet met with envisioned success. Down-regulation of neutral INV in transgenic sugarcane resulted in increased concentrations of stored sucrose and reduced respiration; however, there was a severe negative effect on the overall plant vigor (Rossouw et al., 2010). Further, there was no increase in sucrose levels by constitutively expressing a SPS gene in sugarcane (Vickers et al., 2005). This may suggest that SPP was limiting and that both enzymes need to be coordinately up-regulated to achieve increases in sucrose content. Still, recent breakthroughs have been attained by manipulating sugar levels in sugarcane stems through transgenic approaches. Sugarcane expressing a sucrose isomerase enzyme in stem vacuoles resulted in plants with "near-complete" conversion of sucrose in mature internodes and production of novel metabolites (Wu and Birch, 2007, 2010), although some of the sugar increases attained in greenhouse conditions were not realized in the field (Basnayake et al., 2012; Patrick et al., 2013).

Another possible approach is the manipulation of sucrose transporters, such as SUTs or SWEETs, which could lead to an enhanced sucrose flow from phloem tissues into stem storage cells, particularly in sweet sorghum (**Figure 1C**). However, because

of the limited data on the function of the known transporters (Braun and Slewinski, 2009), more work is needed before targeting these membrane proteins for genetic improvement in sugarcane or sweet sorghum. For example, the heterologous overexpression of a spinach (*Spinacia oleracea*) SUT in potato resulted in tubers with increased sugar content and reduced amino acid levels, but yielded tubers with very minimal changes in the starch content and biomass (Leggewie et al., 2003). Further, as SWEET genes have been identified as the targets of microbial pathogens to obtain sugars from plants (Chu et al., 2006; Yang et al., 2006; Chen et al., 2010; Baker et al., 2012; Yuan and Wang, 2013), novel strategies will need to be employed in targeting these genes for genetic engineering to avoid creating highly susceptible crops (Li et al., 2012). As another potential approach for genetic engineering, SUTs have been reported to be regulated by protein phosphorylation (Ransom-Hodgkins et al., 2003; Niittyla et al., 2007). Unfortunately, the responsible protein kinases and protein phosphatases are still unknown. In a different approach, overexpression of TMT1 in *Arabidopsis thaliana* resulted in plants with increased activity in photosynthesis-related genes, decreased sugar use for respiration, and increased capacity for sugar export from source leaves (Wingenter et al., 2010). Hence, TMT1 may represent an attractive molecular target. To our knowledge, the strategy of manipulating the functions of sucrose transporters using transgenic approaches in crops that accumulate sucrose in their stems, such as sugarcane and sweet sorghum, has not been reported.

Because of the economic importance of stored sucrose in the stem, increasing its yield presents a valuable target for plant biotechnologists. However, as indicated above, stem sucrose improvement using transgenic approaches is very challenging. A successful and highly efficient transformation protocol for sweet sorghum is yet to be designed, and in the case of sugarcane, transformation is difficult due to the high rate of gene silencing (Mudge et al., 2009; Merwe et al., 2010). Significant improvement of sugarcane have been reached using conventional breeding, but this is challenging due to its complex polyploid genome (Waclawovsky et al., 2010). Nevertheless, there is potential to increase sugar levels in the stem through metabolic engineering, but we need to expand our understanding of plant sugar metabolism and transport before undertaking such an effort (Patrick et al., 2013). The tantalizing possibility of further improving sweet sorghum and sugarcane stem sucrose content is at hand if we can understand the mechanisms that regulate stem sink strength.

MOLECULAR CONTRIBUTORS TO STRONG SINK STRENGTH IN GRAINS

The maize kernel during starch-deposition has long been a system of focus for analysis of carbon movement into grains (Sabelli and Larkins, 2009). A classical model for kernel sink strength is outlined by the Shannon hypothesis for cleavage and resynthesis of sucrose entering the maize endosperm (**Figure 2**; Shannon, 1972). In this scenario, sucrose is metabolized by a combination of cell wall and vacuolar INVs in the phloem-unloading zone, and resynthesized in the endosperm prior to starch biosynthesis (Shannon, 1972; Shannon and Dougherty, 1972; Felker and Shannon, 1980). The model is supported by diverse lines of evidence extending from radiotracer studies (Shannon, 1972; Felker and Shannon, 1980), to enzyme activity (Shannon and Dougherty, 1972; Im, 2004; Koch, 2004), mRNA levels (Koch, 1996; Xu et al., 1996), mutant phenotypes (Vilhar et al., 2002; Keeling and Myers, 2010), and environmental perturbations (Andersen et al., 2002; Setter and Parra, 2010). A prominent role for INV in basal regions of the kernel is consistent with proposed advantages to phloem unloading that would enhance overall turgor gradients and pressure-driven movement of sucrose in the phloem (Turgeon, 2010). INV action in this zone has also been proposed to enhance sugar signals important to establishment of young sinks (see below). Although sucrose cleavage is not required for sucrose entry into the developing endosperm (Schmalstig and Hitz, 1987), INV clearly has a major role in the initial metabolism and signaling of sugars entering kernels (Koch, 2004; Ruan et al., 2010; Aoki et al., 2012).

The path of sucrose transfer and metabolism in kernels and other grains has thus emerged as a central feature of their sink strength. Disruptions to any of the steps involved can have major consequences for the capacity to import and use sucrose. **Figure 2** diagrams a current view of maize kernel sink strength. Evidence for each of their roles includes a range of genetic, transgenic, and environmental alterations, beginning with decreases in activities of the sucrose-metabolizing vacuolar INV (Andersen et al., 2002) and cell wall INV (Vilhar et al., 2002) that severely curtail kernel development. Neutral INV involvement has been enigmatic (Huang et al., 1992). Overall assimilate import is also reduced by disruptions to the second path of sucrose cleavage mediated by the reversible SUS reaction (Koch, 2004; Aoki et al., 2012). Sucrose resynthesis in endosperm is well-documented (Shannon, 1972; Hennen-Bierwagen and Myers, 2013), and evidence supports contributions by SPS and SPP to sink strength of grains (Im, 2004; Sharma et al., 2010) and other fruits (Nguyen-Quoc et al., 1999).

Final storage products are the ultimate sinks, and numerous mutations to genes for starch biosynthesis show that this process affects not only the composition of grains and kernels, but also their sink strength (Creech, 1965; Hennen-Bierwagen and Myers, 2013). In rice (*Oryza sativa*), starch biosynthesis is suppressed and amylopectin structure altered if a negative regulator of starch synthesis, *Rice Starch Regulator1* (*RSR1*), is overexpressed. Conversely, when *RSR1* is down-regulated, starch production increases, seed size rises, and overall yield is also greater (Fu and Xue, 2010). Disruption to starch biosynthesis typically elevates sugar levels [as in sweet corn (Creech, 1965; Hennen-Bierwagen and Myers, 2013)], which has far-reaching effects on sugar signals to other metabolic and developmental programs (Koch, 1996, 2004; Ruan et al., 2010; Aoki et al., 2012). Genetic analyses of such mutants in barley (*Hordeum vulgare*), for example, revealed that alteration of ADP-glucose pyrophosphorylase (AGPase) was accompanied by co-regulation of multiple genes for starch biosynthesis, glycolysis, amino acid storage, and sugar/ABA sensing (Faix et al., 2012). In this way, reduced flux into starch biosynthesis led to induction of mechanisms for decreasing sugar accumulation (and accompanying potential for oxidative stress; Faix et al., 2012).

Minimally understood, but potentially influential components of the post-phloem path into kernels are transporters and efflux constituents (Braun, 2012; Patrick et al., 2013). Their involvement is implicated by apoplastic steps of post-phloem transfer in maize kernels, but symplastic roles of these transporters can also include

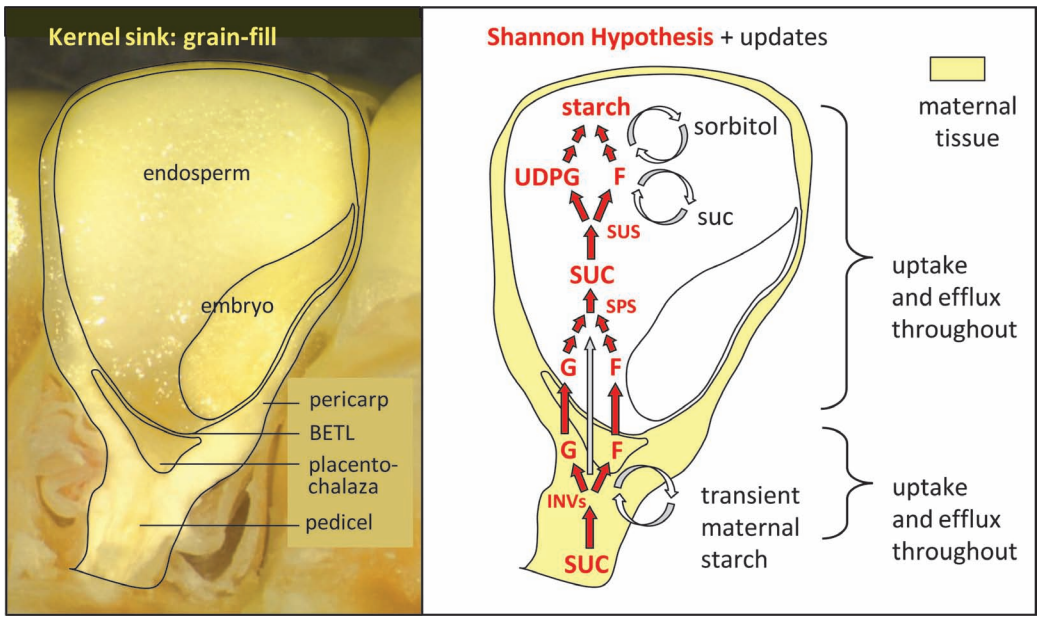

FIGURE 2 | Maize kernel sink strength during grain fill. The left panel shows a fresh, longitudinal view of a maize kernel during active starch deposition in the upper endosperm and lower pericarp (12 days after pollination). The path of assimilate entry into endosperm or embryo includes mandatory movement through the cell wall space (apoplast), because these tissues are physically isolated from the maternal structures around them by a lack of plasmodesmatal (symplastic) continuity. The right panel diagrams major contributors to sink strength relative to sites of their spatial distribution in kernels at this stage. The original Shannon hypothesis is shown in red, with updates in black. Maternal tissues are shown in yellow to emphasize the role of apoplastic transfer. Sucrose (SUC) is first cleaved by vacuolar and cell wall invertases (INV) operating in the pedicel, the placenta-chalaza, and especially by cell wall INV in the basal endosperm transfer layer (BETL). Updates to the first portion of this classical path include continual influx and efflux from maternal cells (via transporters and effluxers), and a prominent role for transient starch reserves in maternal tissues (especially the lower pericarp). Assimilates next enter the endosperm across the BETL primarily as hexoses, glucose (G) and fructose (F), but also as SUC. SUC is also resynthesized in basal portions of the endosperm by sucrose phosphate synthase (SPS), and transported to upper regions of the endosperm. Recent evidence shows extremely low oxygen levels in endosperm, which can affect several aspects of kernel sink strength. These include a limitation to metabolism of stored assimilates, advantages of sucrose resynthesis and cleavage by sucrose synthase [SUS (typically cleaving sucrose *in vivo*)], and cycling of sorbitol as a mechanism of redox balance under deep hypoxia. UDPG, uridine-di-phosphoglucose.

vesicle-based transfer processes (Echeverría, 2000; Lalonde et al., 2004), transient storage by vacuoles (Eom et al., 2011), cellular efflux (Lalonde et al., 2004; Chen et al., 2010, 2012; Baker et al., 2012; Braun, 2012), or action as sensors (Kang and Turano, 2003; Lalonde et al., 2004; Slewinski, 2011; Aoki et al., 2012; Price et al., 2012). In maize kernels, prominent contributions are indicated for both sucrose and hexose transporters (Aoki et al., 2012), and the importance of SWEET-mediated efflux to diverse sinks (Chen et al., 2010, 2012), led to suggested roles in efflux to seeds (Braun, 2012). Maize SWEETs include paralogs expressed most prominently in the basal endosperm transfer layer (BETL; Xiong et al., 2011).

Other potential determinants of sink strength in kernels include not only genes for diverse aspects of both carbon and nitrogen metabolism (Koch, 1997; Coruzzi and Bush, 2001; Uribelarrea et al., 2004; Seebauer et al., 2010) but also those affecting cell wall biosynthesis and expansion (Shevell et al., 2000; Lizana et al., 2010; Manavski et al., 2012; Liu et al., 2013). Although starch is a prominent end-product of assimilates imported into maize kernels, so too is the oil stored in the embryo scutellum (Rolletschek et al., 2005; Shen et al., 2010). Embryo size, its oil content, and carbon partitioning between embryo and endosperm can all be altered at the genetic level in maize (Shen et al., 2010; Yang et al., 2010). Oil biosynthesis and deposition can thus be a significant contributor to strong sink strength in maize kernels. The role of nitrogen in carbon sink strength of kernels may be more complex, since formation of storage proteins can vary in its relation to starch deposition (Singletary et al., 1990; Kang and Turano, 2003; Uribelarrea et al., 2004; Wang et al., 2011). An interface between carbon/nitrogen import is also evident at other levels, including amino acid sensing (Kang and Turano, 2003; Seebauer et al., 2004, 2010), often reciprocal responses to carbon and nitrogen abundance by individual genes (Koch, 1997), limitations to capacity for carbon import in female florets of nitrogen-deficient plants (Peng et al., 2012), and a range of nitrogen effects on kernel sinks (Singletary et al., 1990; Uribelarrea et al., 2004).

A previously unexpected role of low oxygen has emerged for sink strength of maize endosperm, which normally has very low oxygen levels (Rolletschek et al., 2005). Deep hypoxia appears to be a common feature of starch-storing endosperms such as that of barley (Rolletschek et al., 2011), but not of the adjacent embryos (Rolletschek et al., 2005). If this deep hypoxia is indeed characteristic of diverse grain endosperms, then deposition of starch and protein in the world's major grain crops is likely to occur in a very different endogenous environment than was recognized earlier. Although the outermost endosperm cells are partially oxygenated

in smaller grains (Rolletschek et al., 2011), oxygen levels drop to limits of detection immediately inside the pericarp of maize kernels (Rolletschek et al., 2005). Metabolite analyses and modeling studies of kernels concur with predicted, low oxygen alterations to glycolysis, redox status, and other effectors of sink strength (Koch et al., 2000; Méchin et al., 2004; Rolletschek et al., 2005; Grafahrend-Belau et al., 2009; Rolletschek et al., 2011). Implications extend to pyrophosphate (PPi) cycling, and its potential role in balancing starch deposition with glycolytic rate. In a low oxygen endosperm, ATP limitations would favor PPi-driven glycolysis [via PPi-phosphofructokinase (Huber and Akazawa, 1986; Zeng et al., 1998; Koch, 2004)], and this demand for PPi (together with that of UDP-glucose pyrophosphorylase) could be met and balanced by the PPi produced during starch biosynthesis by AGPase. Deep hypoxia in endosperm, rather than being a stress, may thus be advantageous to starch biosynthesis and ultimate sink strength.

Sink strength of newly forming kernels is determined differently (**Figure 3**), and can affect which will abort and which will not. The first third of kernel development is vital to establishment of kernel number, since this is the period when maternal "decisions" are made regarding seed-sink load (Setter and Parra, 2010). Until this stage, a young maize grain is largely maternal tissues (nucellus and pericarp) that import sucrose by mechanisms distinct from those of more mature grains (endosperm and embryo; Sabelli and Larkins, 2009; Aoki et al., 2012). These maternal cells of young maize kernels and other grains are symplastically continuous, actively dividing, and rapidly expanding, yet imported assimilates move into both the apoplast and symplast of pre-pollination ovaries (Sabelli and Larkins, 2009; Aoki et al., 2012; Tang and Boyer, 2013). Both vacuolar and cell wall INVs are prominent in provision of hexoses in these organs, not only for downstream carbon metabolism, but also for the hexose-based sugar signals important to up-regulation of sink strength in young organs (Andersen et al., 2002; Koch, 2004; Ruan et al., 2010). Effects include genes for sucrose import, cell cycle regulation, and establishment of new sinks (Koch, 2004; Ruan et al., 2010). Many of the mutations responsible for the most devastating kernel phenotypes are ones that alter sink strength at this early stage of development, through disruption of carbon use in respiration (Manavski et al., 2012; Liu et al., 2013), cell wall biosynthesis (Shevell et al., 2000), or sugar signals to other genes (Koch, 2004; Ruan et al., 2010; Aoki et al., 2012).

Molecular features of strong sinks in small-grain pooid species may well differ, due to the distinctive features of this group compared to the panicoids. The panicoids (e.g., maize, sugarcane, and sorghum) typically use C_4 photosynthesis and have a maize-type grain structure, whereas the pooids [e.g., rice, wheat (*Triticum aestivum*), and barley] are C_3 species with a rice-type grain structure. Although not universal, overall source–sink relations in pooids often show a trade-off between seed size and seed number that implies a source-limited system (Acreche and Slafer, 2006). The same appears less evident for C_4 panicoids such as maize, however, which are typically large, with high capacities for carbon assimilation (Borrás et al., 2004; McCormick et al., 2009). The panicoid and pooid grain structures also differ (Hands et al., 2012), and studies of assimilate entry into them are consistent with different contributions by key determinants of sink strength (Rolletschek et al., 2005, 2011; Aoki et al., 2012).

MOLECULAR BASIS FOR STRONG SINK STRENGTH IN OTHER FRUITS, SEEDS, AND FLOWERS

The biosynthesis and regulation of cell walls may be central to strong sinks in many organs. Young structures in particular, often have high rates of cell division (Boisnard-Lorig et al., 2001) that involve significant allocation of carbon to biosynthesis of new cell walls. Later, during cell expansion, sink strength

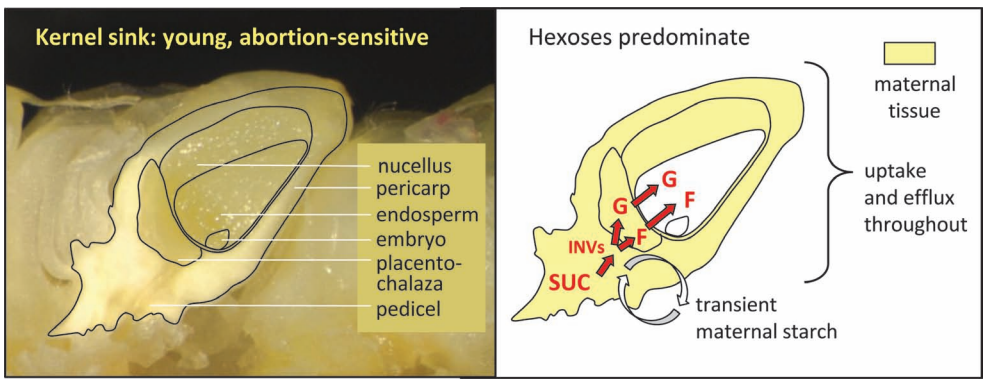

FIGURE 3 | Maize kernel sink strength in young, abortion-sensitive ovaries. The left panel shows a fresh, longitudinal view of a maize kernel at eight days after pollination, when maternal tissues predominate in this sink structure. Note the importance of the nucellus and expanding pericarp (fruit wall). The right panel diagrams major contributors to sink strength relative to their spatial distribution. Maternal tissues are shown in yellow to emphasize their dominant role in sink strength, and control over abortion and kernel set at this stage. Like the more mature kernels, sucrose (SUC) in young ovaries is first cleaved by vacuolar and cell wall invertases (INVs) in the pedicel and the placenta-chalaza, and to some extent the newly forming basal endosperm transfer layer. Updates have indicated that collective effects of these INVs are most pronounced in very young kernels, where the hexoses have signaling roles in the cell cycle, cell division, and cell number that markedly enhance ultimate sink strength. Another component of young-kernel sink strength is transient maternal starch, thought to aid maintenance of non-aborting kernels. In addition, continuous efflux and influx of sugars occur throughout maternal tissues, even prior to pollination, highlighting the roles for transporters and effluxers during early development of sink strength in maize kernels. G, glucose; F, fructose.

can also be affected by regulation of cell wall properties, such as the combined relaxation and new synthesis that facilitate turgor-driven expansion (Cosgrove, 2005). This aspect of sink strength can be especially important for accommodating phloem water in minimally transpiring sink organs. Cell wall loosening signals and enzymes can thus be critical to a tissue's sink strength. Various cell wall enzymes and proteins have been implicated in the loosening that occurs during cell growth, including expansins (Fleming et al., 1997; Link and Cosgrove, 1998) and xyloglucan endotransglucosylase/hydrolases (Fry et al., 1992).

In addition to their structural roles, non-cellulosic cell wall polysaccharides can also be major storage constituents in many fruits and seeds (Buckeridge, 2010). In this respect their contribution to sink strength may be analogous to that of starch. Examples include the xyloglucans in endosperms of legumes such as nasturtium (*Tropaeolum majus*; Edwards et al., 1985), the galactomannans in seeds such as fenugreek (*Trigonella foenum-graecum*; Reid and Meier, 1972; Reid et al., 1977), and the β-glucans and arabinoxylans that appear early in developing endosperms of wheat and barley (Meier and Reid, 1982; Philippe et al., 2006). In the starchy endosperm of barley, β-glucans represent 70% of wall material at maturation (Fincher, 1975; Wilson et al., 2006), and together with arabinoxylans [which comprise 85% of the aleurone wall (McNeil et al., 1975)], provide 18.5% of the carbon used by germinating embryos (Morrall and Briggs, 1978). The sink strength of developing seeds, as well as their subsequent germination, may also be aided by the water-holding features of arabinoxylans [100 g H_20 g^{-1} polymer (Izydorczyk and Biliaderis, 1995)]. This 100-to-1 ratio of water-to-polysaccharide (g/g) indicates a capacity for arabinoxylans to aid handling of the excess water entering sinks that have reached their full size and are no longer transpiring (this can otherwise affect assimilate import; Koch and Avigne, 1990; Huang et al., 1992; Zhang et al., 2007; Turgeon, 2010).

Diverse molecular contributors to sink strength emerge in analyses of different plant systems. Overall import of photosynthates can be increased in a number of structures and species, for example, by up-regulating genes for starch biosynthesis (Wang et al., 2007; Baroja-Fernández et al., 2009; Li et al., 2011, 2013). Also, cell cycle regulation can make important differences to sink strength, as evidenced by overexpression of a D-type cyclin gene in developing seeds of Arabidopsis. Resulting seeds were larger, cell number and size were greater, and growth was increased for both embryo and endosperm (Collins et al., 2012).

Strong floral sinks are vital for establishment of the fruits they will become. Rapid cell division and expansion are critically important to virtually all floral parts, and especially the structures involved in pollination (Xu et al., 1996; Cosgrove, 2005; Aoki et al., 2012; Reeves et al., 2012). Where showy petals are produced, INVs often determine blossom size and floral sink strength (Gübitz et al., 2009). Nectary structures also vary markedly, but again, involve genes for INV, vacuolar compartmentalization, and secretory functions (Ge et al., 2000; Gübitz et al., 2009; Chen et al., 2010). Pollination success in maize requires an expansin- and INV-mediated elongation of the floral silk (a stigmatic style), which is one of the most rapidly elongating structures in the plant kingdom (Xu et al., 1996; Cosgrove, 2005). Similar INV requirements are evident for anthers of rice (Ruan et al., 2010). Expansion and sink strength of pre-pollination ovaries also depend on INVs that are sugar- and stress-sensitive determinants of later sink strength (Xu et al., 1996; Andersen et al., 2002; Koch, 2004; Lizana et al., 2010). Transgenic approaches to increase maize yields are also targeting the enhancement of INV activity and/or starch biosynthesis in florets (Tomes et al., 2011). These centrally important aspects of expansion are also mediated by auxin, which is critical to the earliest stages of sink strength development in flowers (Barazesh et al., 2009; Phillips et al., 2011; Reeves et al., 2012).

Expansion is also a prominent contributor to sink strength of fleshy fruit, regardless of whether a given fruit stores starch or soluble sugars. The strongest sinks among young fruit typically have the highest INV activity, greatest respiration rate, and most rapid cell division (Huang et al., 1992; Koch, 2004; Ruan et al., 2012b). Central contributions have also been attributed to sugar transporters (Zhang et al., 2007; Slewinski, 2011; Aoki et al., 2012), and are suggested for SWEET-mediated efflux in fruit (Baker et al., 2012; Braun, 2012). Roles of auxin and gibberellins parallel those noted above for floral sinks (Aoki et al., 2012). Water handling becomes a key feature for those organs importing sugars without transpiring or expanding [e.g., citrus fruit during most of their development (Koch and Avigne, 1990; Huang et al., 1992)], since excess phloem water can become a problem for such sinks (Huang et al., 1992; Zhang et al., 2007; Turgeon, 2010).

FEAST-OR-FAMINE SIGNALS AND FEED-FORWARD ENHANCEMENT OF SINK STRENGTH

Sugar-responsiveness is a prominent feature of genes contributing to sink strength of developing organs, and provides an important mechanism for sink adjustment to source supplies (Koch, 1996; Koch et al., 2000; Ramon et al., 2008; Ruan et al., 2010; Tiessen and Padilla-Chacon, 2013; Xiong et al., 2013). Sink genes up-regulated by sugars span the full spectrum of those involved in sucrose import and use. They range from sucrose cleavage to deposition of storage products (Li et al., 2002; Smidansky et al., 2002; Ramon et al., 2008; Kang et al., 2010). Similar regulation occurs at multiple levels from transcription (Koch, 1996, 2004; Li et al., 2002; Ramon et al., 2008) to translation (Zeng et al., 1998), and from mRNA longevity (Zeng et al., 1998; Koch, 2004; Huang et al., 2007) to protein turnover (Koch, 1996, 2004; Huang et al., 2007). Multiple paths of sugar signaling are also involved, such that sucrose can be sensed differently from hexoses, and endogenous metabolic flux can be sensed differently than exogenous sugars (Koch, 1996; Ruan et al., 2010; Tiessen and Padilla-Chacon, 2013). The end result is a means by which strongest sinks can be maximally up-regulated through input from greatest photosynthate supplies [The reverse would also allow a balanced down-regulation (Kang et al., 2010)].

Still further enhancement of sink strength can occur through an INV-mediated, feed-forward process that increases hexose production, and thus those sugar signals that arise from hexose-based sensing (Koch, 1996, 2004; Huang et al., 2007; Ruan et al., 2010; Tiessen and Padilla-Chacon, 2013). Multiple hexose-based avenues of sugar signaling are known effectors of genes for the cell cycle, hormone interaction, and others important to development

of new sinks (Koch, 1996, 2004; Rolland et al., 2006; Ramon et al., 2008; Kang et al., 2010; Ruan et al., 2010; Moghaddam and Van den Ende, 2013). The same process may also be involved in initiation of new sinks through demonstrated interactions with phytochrome sensing (Kang et al., 2010; Moghaddam and Van den Ende, 2013), low oxygen signals (Koch et al., 2000; Koch, 2004), and hormonal control of meristem fate (Francis and Halford, 2006). In developing seeds and fruits, a predominance of hexoses is often associated with cell division and expansion, whereas elevated sucrose levels coincide with differentiation and maturation (Koch and Avigne, 1990; Weber et al., 1997; Sabelli and Larkins, 2009; Ruan et al., 2012b).

These changes are compatible with a "feast-or-famine" model for adjustment of source–sink relations in plants (Koch, 1996; Coruzzi and Bush, 2001; Mandadi et al., 2009), a framework initially proposed for understanding sugar responses by multicellular organisms (Koch, 1996). A highly conserved, ancient system is involved, and in microorganisms it constitutes an essential means of sensing and acclimating to the nutrient environment (e.g., the *lac* operon and others; Yokoyama et al., 2006). Maximal sink strength of plant organs will thus include "feast" genes that respond to carbohydrate abundance and enhance its use. Similarly, source capacity will increase when enhanced sink demands for photosynthate reduce sugar levels in leaves and up-regulate expression of "famine" genes for acquisition of the limited carbon resources (e.g., de-repression of photosynthetic genes). Indirect effects of enhanced sink strength therefore include higher rates of photosynthesis in diverse systems due to release of feed-back inhibition at a metabolic level and gene repression at a transcriptional level (Koch, 1996, 2004; Ramon et al., 2008; Kang et al., 2010). Greater overall productivity and yield is thus a reasonable prediction for instances where sink strength can be increased.

CONCLUDING REMARKS

Increased demand for food and energy security compels researchers to investigate different avenues to improve plants to meet our needs. Carbohydrates stored in non-photosynthetic sink organs are vital to humans as food, feed, fuel, and for other industrial uses; therefore, understanding the functions of the molecular drivers of sink strength is essential to enhance the capability of sink organs to import photoassimilates. Some of the sucrose transport pathways and important players for carbohydrate storage and utilization have been identified. However, future research should focus on examining and understanding the exact roles of different sugar transporters, metabolic control of carbohydrate storage in sinks, sugar signaling regulating sink strength, and long-distance communication coordinating carbohydrate partitioning between sink and source tissues. For example, it is not clear how sucrose transporters, such as SWEETs, contribute to sink strength. Furthermore, it remains to be shown whether their genetic manipulation will enhance the delivery of photoassimilates to grains, seeds, and fruits for food, or, in the case of sweet sorghum and sugarcane, to stems for biofuel production. Detailed genetic, biochemical, and physiological analyses, in addition to molecular and genomic investigations, will facilitate the identification and characterization of novel players involved in defining sink strength, and will provide a deeper knowledge of how plants regulate whole-plant carbohydrate partitioning.

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REFERENCES

Acreche, M. M., and Slafer, G. A. (2006). Grain weight response to increases in number of grains in wheat in a Mediterranean area. *Field Crops Res.* 98, 52–59. doi: 10.1016/j.fcr.2005.12.005

Ainsworth, E. A., and Bush, D. R. (2011). Carbohydrate export from the leaf: a highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiol.* 155, 64–69. doi: 10.1104/pp.110.167684

Aitken, K. S., Hermann, S., Karno, K., Bonnett, G. D., McIntyre, L. C., and Jackson, P. A. (2008). Genetic control of yield related stalk traits in sugarcane. *Theor. Appl. Genet.* 117, 1191–1203. doi: 10.1007/s00122-008-0856-6

Ali, M. L., Rajewski, J. F., Baenziger, P. S., Gill, K. S., Eskridge, K. M., and Dweikat, I. (2008). Assessment of genetic diversity and relationship among a collection of US sweet sorghum germplasm by SSR markers. *Mol. Breed.* 21, 497–509. doi: 10.1007/s11032-007-9149-z

Almodares, A., and Hadi, M. R. (2009). Production of bioethanol from sweet sorghum: a review. *Afr. J. Agric. Res.* 4, 772–780.

Almodares, A., Taheri, R., and Adeli, S. (2008). Stalk yield and carbohydrate composition of sweet *Sorghum* (*Sorghum bicolor* (L.) Moench) cultivars and lines at different growth stages. *Malays. Appl. Biol.* 37, 31–36.

Andersen, C. P. (2003). Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytol.* 157, 213–228. doi: 10.1046/j.1469-8137.2003.00674.x

Andersen, M. N., Asch, F., Wu, Y., Jensen, C. R., Naested, H., Mogensen, V. O., et al. (2002). Soluble invertase expression is an early target of drought stress during the critical, abortion-sensitive phase of young ovary development in maize. *Plant Physiol.* 130, 591–604. doi: 10.1104/pp.005637

Aoki, N., Hirose, T., and Furbank, R. T. (2012). "Sucrose transport in higher plants: from source to sink," in *Photosynthesis: Plastid Biology, Energy Conversion, and Carbon Assimilation, Advances in Photosynthesis and Respiration*, eds J. J. Eaton-Rye, B. C. Tripathy, and T. D. Sharkey (Dordecht: Springer), 703–729.

Aoki, N., Hirose, T., Takahashi, S., Ono, K., Ishimaru, K., and Ohsugi, R. (1999). Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (*Zea mays* L.). *Plant Cell Physiol.* 40, 1072–1078. doi: 10.1093/oxfordjournals.pcp.a029489

Aranjuelo, I., Cabrera-Bosquet, L., Morcuende, R., Avice, J. C., Nogués, S., Araus, J. L., et al. (2011). Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO2? *J. Exp. Bot.* 62, 3957–3969. doi: 10.1093/jxb/err095

Artschwager, E. (1948). Anatomy and morphology of the vegetative organs of *Sorghum vulgare*. US Department of Agriculture, Washington, D.C., Technical Bulletin No. 957.

Ayre, B. G. (2011). Membrane-transport systems for sucrose in relation to whole-plant carbon partitioning. *Mol. Plant* 4, 377–394. doi: 10.1093/mp/ssr014

Baker, R. F., Leach, K. A., and Braun, D. M. (2012). SWEET as sugar: new sucrose effluxers in plants. *Mol. Plant* 5, 766–768. doi: 10.1093/mp/SSS054

Barazesh, S., Nowbakht, C., and McSteen, P. (2009). sparse inflorescence1, barren inflorescence1 and barren stalk1 promote cell elongation in maize inflorescence development. *Genetics* 182, 403–406. doi: 10.1534/genetics.108.099390

Baroja-Fernández, E., Muñoz, F. J., Montero, M., Etxeberria, E., Sesma, M. T., Ovecka, M., et al. (2009). Enhancing sucrose synthase activity in transgenic potato (*Solanum tuberosum* L.) tubers results in increased levels of starch, ADPglucose and UDPglucose and total yield. *Plant Cell Physiol.* 50, 1651–1662. doi: 10.1093/pcp/pcp108

Basnayake, S. W. V., Morgan, T. C., Wu, L., and Birch, R. G. (2012). Field performance of transgenic sugarcane expressing isomaltulose synthase.

Plant Biotechnol. J. 10, 217–225. doi: 10.1111/j.1467-7652.2011.00655.x

Batta, S. K., and Singh, R. (1986). Sucrose metabolism in sugarcane grown under varying climatic conditions: synthesis and storage of sucrose in relation to the activities of sucrose synthase, sucrose-phosphate synthase and invertase. *Phytochemistry* 25, 2431–2437. doi: 10.1016/S0031-9422(00)84484-2

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., et al. (2001). Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. *Plant Cell* 13, 495–509.

Borrás, L., Slafer, G. A., and Otegui, M. E. (2004). Seed dry weight response to source–sink manipulations in wheat, maize and soybean: a quantitative reappraisal. *Field Crops Res.* 86, 131–146.

Botha, F. C., and Black, K. G. (2000). Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. *Funct. Plant Biol.* 27, 81–85. doi: 10.1071/PP99098

Braun, D. M. (2012). SWEET! The pathway is complete. *Science* 335, 173–174. doi: 10.1126/science.1216828

Braun, D. M., and Slewinski, T. L. (2009). Genetic control of carbon partitioning in grasses: roles of sucrose transporters and tie-dyed loci in phloem loading. *Plant Physiol.* 149, 71–81. doi: 10.1104/pp.108.129049

Buckeridge, M. S. (2010). Seed cell wall storage polysaccharides: models to understand cell wall biosynthesis and degradation. *Plant Physiol.* 154, 1017–1023. doi: 10.1104/pp.110.158642

Calviño, M., and Messing, J. (2012). Sweet sorghum as a model system for bioenergy crops. *Curr. Opin. Biotechnol.* 23, 323–329. doi: 10.1016/j.copbio.2011.12.002

Calviño, M., Miclaus, M., Bruggmann, R., and Messing, J. (2009). Molecular markers for sweet sorghum based on microarray expression data. *Rice* 2, 129–142.

Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X.-Q., et al. (2010). Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468, 527–532. doi: 10.1038/nature09606

Chen, L. Q., Qu, X. Q., Hou, B. H., Sosso, D., Osorio, S., Fernie, A. R., et al. (2012). Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* 335, 207–211. doi: 10.1126/science.1213351

Chu, Z., Yuan, M., Yao, J., Ge, X., Yuan, B., Xu, C., et al. (2006). Promoter mutations of an essential gene for pollen development result in disease resistance in rice. *Genes Dev.* 20, 1250–1255. doi: 10.1101/gad.1416306

Collins, C., Dewitte, W., and Murray, J. A. (2012). D-type cyclins control cell division and developmental rate during *Arabidopsis* seed development. *J. Exp. Bot.* 63, 3571–3586. doi: 10.1093/jxb/ers015

Coruzzi, G., and Bush, D. R. (2001). Nitrogen and carbon nutrient and metabolite signaling in plants. *Plant Physiol.* 125, 61–64. doi: 10.1104/pp.125.1.61

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861. doi: 10.1038/nrm1746

Creech, R. G. (1965). Genetic control of carbohydrate synthesis in maize endosperm. *Genetics* 52, 1175.

Creste, S., Accoroni, K. G., Pinto, L., Vencovsky, R., Gimenes, M., Xavier, M., et al. (2010). Genetic variability among sugarcane genotypes based on polymorphisms in sucrose metabolism and drought tolerance genes. *Euphytica* 172, 435–446. doi: 10.1007/s10681-009-0078-2

Echeverría, E. (2000). Vesicle-mediated solute transport between the vacuole and the plasma membrane. *Plant Physiol.* 123, 1217–1226.

Edwards, M., Dea, I. M., Bulpin, P., and Reid, J. S. G. (1985). Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus* L. seeds following germination. *Planta* 163, 133–140. doi: 10.1007/BF00395907

Eom, J.-S., Cho, J.-I., Reinders, A., Lee, S.-W., Yoo, Y., Tuan, P. Q., et al. (2011). Impaired function of the tonoplast-localized sucrose transporter in rice, OsSUT2, limits the transport of vacuolar reserve sucrose and affects plant growth. *Plant Physiol.* 157, 109–119. doi: 10.1104/pp.111.176982

Faix, B., Radchuk, V., Nerlich, A., Hümmer, C., Radchuk, R., Emery, R., et al. (2012). Barley grains, deficient in cytosolic small subunit of ADP-glucose pyrophosphorylase, reveal coordinate adjustment of C:N metabolism mediated by an overlapping metabolic-hormonal control. *Plant J.* 69, 1077–1093. doi: 10.1111/j.1365-313X.2011.04857.x

Felker, F. C., and Shannon, J. C. (1980). Movement of 14C-labeled assimilates into kernels of *Zea mays* L: III. An anatomical examination and microautoradiographic study of assimilate transfer. *Plant Physiol.* 65, 864–870. doi: 10.1104/pp.65.5.864

Fincher, G. B. (1975). Morphology and chemical composition of barley endosperm cell walls. *J. Inst. Brew.* 81, 116–112. doi: 10.1002/j.2050-0416.1975.tb03672.x

Fleming, A. J., McQueen-Mason, S., Mandel, T., and Kuhlemeier, C. (1997). Induction of leaf primordia by the cell wall protein expansin. *Science* 276, 1415–1418. doi: 10.1126/science.276.5317.1415

Francis, D., and Halford, N. G. (2006). Nutrient sensing in plant meristems. *Plant Mol. Biol.* 60, 981–993. doi: 10.1007/s11103-005-5749-3

Fry, S., Smith, R., Renwick, K., Martin, D., Hodge, S., and Matthews, K. (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* 282, 821.

Fu, F.-F., and Xue, H.-W. (2010). Coexpression analysis identifies Rice Starch Regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. *Plant Physiol.* 154, 927–938. doi: 10.1104/pp.110.159517

Ge, Y.-X., Angenent, G. C., Wittich, P. E., Peters, J., Franken, J., Busscher, M., et al. (2000). NEC1, a novel gene, highly expressed in nectary tissue of *Petunia hybrida*. *Plant J.* 24, 725–734. doi: 10.1046/j.1365-313x.2000.00926.x

Gifford, R. M., and Evans, L. (1981). Photosynthesis, carbon partitioning, and yield. *Annu. Rev. Plant Physiol.* 32, 485–509. doi: 10.1146/annurev.pp.32.060181.002413

Glassop, D., Roessner, U., Bacic, A., and Bonnett, G. D. (2007). Changes in the sugarcane metabolome with stem development. Are they related to sucrose accumulation? *Plant Cell Physiol.* 48, 573–584. doi: 10.1093/pcp/pcm027

Grafahrend-Belau, E., Schreiber, F., Koschützki, D., and Junker, B. H. (2009). Flux balance analysis of barley seeds: a computational approach to study systemic properties of central metabolism. *Plant Physiol.* 149, 585–598. doi: 10.1104/pp.108.129635

Grof, C. P. L., Albertson, P. L., Bursle, J., Perroux, J. M., Bonnett, G. D., and Manners, J. M. (2007). Sucrose-phosphate synthase, a biochemical marker of high sucrose accumulation in sugarcane. *Crop Sci.* 47, 1530–1539. doi: 10.2135/cropsci2006.12.0825

Grof, C. P. L., So, C. T. E., Perroux, J. M., Bonnett, G. D., and Forrester, R. I. (2006). The five families of sucrose-phosphate synthase genes in *Saccharum* spp. are differentially expressed in leaves and stem. *Funct. Plant Biol.* 33, 605–610. doi: 10.1071/FP05283

Gübitz, T., Hoballah, M. E., Dell'Olivo, A., and Kuhlemeier, C. (2009). "*Petunia* as a model system for the genetics and evolution of pollination syndromes," in *Petunia, Evolutionary, Developmental and Physiological Genetics*, eds T. Gerats and J. Strommer (New York: Springer), 29–49.

Hammer, G. L., Dong, Z., McLean, G., Doherty, A. I., Messina, C., Schussler, J., et al. (2009). Can changes in canopy and/or root system architecture explain historical maize yield trends in the U.S. Corn belt? *Crop Sci.* 49, 299–312. doi: 10.2135/cropsci2008.03.0152

Hands, P., Kourmpetli, S., Sharples, D., Harris, R. G., and Drea, S. (2012). Analysis of grain characters in temperate grasses reveals distinctive patterns of endosperm organization associated with grain shape. *J. Exp. Bot.* 63, 6253–6266. doi: 10.1093/jxb/ers281

Hennen-Bierwagen, T. A., and Myers, A. M. (2013). "Genomic specification of starch biosynthesis in maize endosperm," in *Seed Genomics*, ed P.W. Becraft (Ames: John Wiley and Sons, Inc.), 123–137.

Herbers, K., and Sonnewald, U. (1998). Molecular determinants of sink strength. *Curr. Opin. Plant Biol.* 1, 207–216. doi: 10.1016/S1369-5266(98)80106-4

Ho, L. C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39, 355–378. doi: 10.1146/annurev.pp.39.060188.002035

Hoffmann-Thoma, G., Hinkel, K., Nicolay, P., and Willenbrink, J. (1996). Sucrose accumulation in sweet sorghum stem internodes in relation to growth. *Physiol. Plant.* 97, 277–284. doi: 10.1034/j.1399-3054.1996.970210.x

Huang, L.-F., Bocock, P. N., Davis, J. M., and Koch, K. E. (2007). Regulation of invertase: a 'suite' of transcriptional and post-transcriptional mechanisms. *Funct. Plant Biol.* 34, 499–507. doi: 10.1071/FP06227

Huang, T., Darnell, R., and Koch, K. (1992). Water and carbon budgets of developing citrus fruit. *J. Am. Soc. Hortic. Sci.* 117, 287–293.

Huber, S. C., and Akazawa, T. (1986). A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells.

Plant Physiol. 81, 1008–1013. doi: 10.1104/pp.81.4.1008

Im, K. H. (2004). Expression of sucrose-phosphate synthase (SPS) in non-photosynthetic tissues of maize. *Mol. Cells* 17, 404–409.

Izydorczyk, M. S., and Biliaderis, C. G. (1995). Cereal arabinoxylans: advances in structure and physicochemical properties. *Carbohydr. Polym.* 28, 33–48. doi: 10.1016/0144-8617(95)00077-1

Kaiser, G., and Heber, U. (1984). Sucrose transport into vacuoles isolated from barley mesophyll protoplasts. *Planta* 161, 562–568. doi: 10.1007/BF00407090

Kang, J., and Turano, F. J. (2003). The putative glutamate receptor 1.1 (*AtGLR*1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 6872–6877. doi: 10.1073/pnas.1030961100

Kang, S. G., Price, J., Lin, P.-C., Hong, J. C., and Jang, J.-C. (2010). The *Arabidopsis* bZIP1 transcription factor is involved in sugar signaling, protein networking, and DNA binding. *Mol. Plant* 3, 361–373. doi: 10.1093/mp/ssp115

Keeling, P. L., and Myers, A. M. (2010). Biochemistry and genetics of starch synthesis. *Annu. Rev. Food Sci. Technol.* 1, 271–303. doi: 10.1146/annurev.food.102308.124214

Koch, K. E. (1996). Carbohydrate-modulated gene expression in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 509–540. doi: 10.1146/annurev.arplant.47.1.509

Koch, K. E. (1997). "Molecular crosstalk and the regulation of C-and N-responsive genes," in *A Molecular Approach to Primary Metabolism in Higher Plants*, eds C. Foyer and P. Quick (London: Taylor and Francis, Inc.), 105–124.

Koch, K. E. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Curr. Opin. Plant Biol.* 7, 235–246. doi: 10.1016/j.pbi.2004.03.014

Koch, K. E., and Avigne, W. T. (1990). Postphloem, nonvascular transfer in citrus: kinetics, metabolism, and sugar gradients. *Plant Physiol.* 93, 1405–1416. doi: 10.1104/pp.93.4.1405

Koch, K. E., Ying, Z., Wu, Y., and Avigne, W. T. (2000). Multiple paths of sugar-sensing and a sugar/oxygen overlap for genes of sucrose and ethanol metabolism. *J. Exp. Bot.* 51, 417–427. doi: 10.1093/jexbot/51.suppl_1.417

Kühn, C., and Grof, C. (2010). Sucrose transporters of higher plants. *Curr. Opin. Plant Biol.* 13, 1–11. doi: 10.1016/j.pbi.2010.02.001

Lalonde, S., Wipf, D., and Frommer, W. B. (2004). Transport mechanisms for organic forms of carbon and nitrogen between source and sink. *Annu. Rev. Plant Biol.* 55, 341–372. doi: 10.1146/annurev.arplant.55.031903.141758

Leggewie, G., Kolbe, A., Lemoine, R., Roessner, U., Lytovchenko, A., Zuther, E., et al. (2003). Overexpression of the sucrose transporter SoSUT1 in potato results in alterations in leaf carbon partitioning and in tuber metabolism but has little impact on tuber morphology. *Planta* 217, 158–167.

Li, J., Baroja-Fernández, E., Bahaji, A., Muñoz, F. J., Ovecka, M., Montero, M., et al. (2013). Enhancing sucrose synthase activity results in increased levels of starch and ADP-glucose in maize (*Zea mays* L.) seed endosperms. *Plant Cell Physiol.* 54, 282–294.

Li, N., Zhang, S., Zhao, Y., Li, B., and Zhang, J. (2011). Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. *Planta* 233, 241–250. doi: 10.1007/s00425-010-1296-5

Li, T., Liu, B., Spalding, M. H., Weeks, D. P., and Yang, B. (2012). High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* 30, 390–392. doi: 10.1038/nbt.2199

Li, X., Xing, J., Gianfagna, T. J., and Janes, H. W. (2002). Sucrose regulation of ADP-glucose pyrophosphorylase subunit genes transcript levels in leaves and fruits. *Plant Sci.* 162, 239–244. doi: 10.1016/S0168-9452(01)00565-9

Lingle, S. (1989). Evidence for the uptake of sucrose intact into sugarcane internodes. *Plant Physiol.* 90, 6–8. doi: 10.1104/pp.90.1.6

Lingle, S. E. (1999). Sugar metabolism during growth and development in sugarcane internodes. *Crop Sci.* 39, 480–486. doi: 10.2135/cropsci1999.0011183X0039000200030x

Link, B. M., and Cosgrove, D. J. (1998). Acid-growth response and α-expansins in suspension cultures of bright yellow 2 tobacco. *Plant Physiol.* 118, 907–916. doi: 10.1104/pp.118.3.907

Liu, Y.-J., Xiu, Z.-H., Meeley, R., and Tan, B.-C. (2013). Empty pericarp5 encodes a pentatricopeptide repeat protein that is required for mitochondrial RNA editing and seed development in maize. *Plant Cell* 25, 868–883. doi: 10.1105/tpc.112.106781

Lizana, X. C., Riegel, R., Gomez, L. D., Herrera, J., Isla, A., McQueen-Mason, S. J., et al. (2010). Expansins expression is associated with grain size dynamics in wheat (*Triticum aestivum* L.). *J. Exp. Bot.* 61, 1147–1157. doi: 10.1093/jxb/erp380

Lunn, J. E., and MacRae, E. (2003). New complexities in the synthesis of sucrose. *Curr. Opin. Plant Biol.* 6, 208–214. doi: 10.1016/S1369-5266(03)00033-5

Ma, Y., Baker, R. F., Magallanes-Lundback, M., DellaPenna, D., and Braun, D. M. (2008). Tie-dyed1 and sucrose export defective1 act independently to promote carbohydrate export from maize leaves. *Planta* 227, 527–538. doi: 10.1007/s00425-007-0636-6

Manavski, N., Guyon, V., Meurer, J., Wienand, U., and Brettschneider, R. (2012). An essential pentatricopeptide repeat protein facilitates 5′ maturation and translation initiation of rps3 mRNA in maize mitochondria. *Plant Cell* 24, 3087–3105. doi: 10.1105/tpc.112.099051

Mancini, M. C., Leite, D. C., Perecin, D., Bidóia, M. A. P., Xavier, M. A., Landell, M. G. A., et al. (2012). Characterization of the genetic variability of a sugarcane commercial cross through yield components and quality parameters. *Sugar Tech* 14, 119–125.

Mandadi, K. K., Misra, A., Ren, S., and McKnight, T. D. (2009). BT2, a BTB protein, mediates multiple responses to nutrients, stresses, and hormones in *Arabidopsis*. *Plant Physiol.* 150, 1930–1939. doi: 10.1104/pp.109.139220

Marcelis, L. F. (1996). Sink strength as a determinant of dry matter partitioning in the whole plant. *J. Exp. Bot.* 47, 1281–1291. doi: 10.1093/jxb/47.Special_Issue.1281

McCormick, A. J., Cramer, M. D., and Watt, D. A. (2006). Sink strength regulates photosynthesis in sugarcane. *New Phytol.* 171, 759–770. doi: 10.1111/j.1469-8137.2006.01785.x

McCormick, A. J., Watt, D. A., and Cramer, M. D. (2009). Supply and demand: sink regulation of sugar accumulation in sugarcane. *J. Exp. Bot.* 60, 357–364. doi: 10.1093/jxb/ern310

McNeil, M., Albersheim, P., Taiz, L., and Jones, R. L. (1975). The structure of plant cell walls: VII. Barley aleurone cells. *Plant Physiol.* 55, 64–68. doi: 10.1104/pp.55.1.64

Méchin, V., Balliau, T., Château-Joubert, S., Davanture, M., Langella, O., Négroni, L., et al. (2004). A two-dimensional proteome map of maize endosperm. *Phytochemistry* 65, 1609–1618.

Meier, H., and Reid, J. S. G. (1982). "Reserve polysaccharides other than starch in higher plants," in *Plant Carbohydrates I*, eds F. A. Loewus and W. Tanner (Berlin: Springer), 418–471. doi: 10.1007/978-3-642-68275-9_11

Merwe, M., Groenewald, J.-H., Stitt, M., Kossmann, J., and Botha, F. (2010). Downregulation of pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase activity in sugarcane culms enhances sucrose accumulation due to elevated hexose-phosphate levels. *Planta* 231, 595–608. doi: 10.1007/s00425-009-1069-1

Moghaddam, M. R. B., and Van den Ende, W. (2013). Sugars, the clock and transition to flowering. *Front. Plant Sci.* 4:22. doi: 10.3389/fpls.2013.00022

Moore, P., and Cosgrove, D. (1991). Developmental changes in cell and tissue water relations parameters in storage parenchyma of sugarcane. *Plant Physiol.* 96, 794. doi: 10.1104/pp.96.3.794

Morrall, P., and Briggs, D. E. (1978). Changes in cell wall polysaccharides of germinating barley grains. *Phytochemistry* 17, 1495–1502. doi: 10.1016/S0031-9422(00)94628-4

Mudge, S., Osabe, K., Casu, R., Bonnett, G., Manners, J., and Birch, R. (2009). Efficient silencing of reporter transgenes coupled to known functional promoters in sugarcane, a highly polyploid crop species. *Planta* 229, 549–558. doi: 10.1007/s00425-008-0852-8

Murray, S. C., Rooney, W. L., Hamblin, M. T., Mitchell, S. E., and Kresovich, S. (2009). Sweet sorghum genetic diversity and association mapping for brix and height. *Plant Genome* 2, 48–62. doi: 10.3835/plantgenome2008.10.0011

Murray, S. C., Rooney, W. L., Mitchell, S. E., Sharma, A., Klein, P. E., Mullet, J. E., et al. (2008a). Genetic improvement of sorghum as a biofuel feedstock: II. QTL for stem and leaf structural carbohydrates. *Crop Sci.* 48, 2180–2193. doi: 10.2135/cropsci2008.01.0068

Murray, S. C., Sharma, A., Rooney, W. L., Klein, P. E., Mullet, J. E., Mitchell, S. E., et al. (2008b). Genetic improvement of sorghum as a biofuel feedstock: I. QTL for stem sugar and grain nonstructural carbohydrates. *Crop Sci.* 48, 2165–2179. doi: 10.2135/cropsci2008.01.0016

Nguyen-Quoc, B., N'Tchobo, H., Foyer, C. H., and Yelle, S. (1999). Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. *J. Exp. Bot.* 50, 785–791. doi: 10.1093/jxb/50.335.785

Niittyla, T., Fuglsang, A. T., Palmgren, M. G., Frommer, W. B., and Schulze, W. X. (2007). Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of *Arabidopsis*. *Mol. Cell. Proteomics* 6, 1711–1726. doi: 10.1074/mcp.M700164-MCP200

Patrick, J. (1997). Phloem unloading: sieve element unloading and post-sieve element transport. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 191–222. doi: 10.1146/annurev.arplant.48.1.191

Patrick, J. W., Botha, F. C., and Birch, R. G. (2013). Metabolic engineering of sugars and simple sugar derivatives in plants. *Plant Biotechnol. J.* 11, 142–156. doi: 10.1111/pbi.12002

Pecina-Quintero, V., Anaya-López, J. L., Zamarripa-Colmenero, A., Montes-García, N., Nuñez-Colín, C., Solis-Bonilla, J. L., et al. (2012). Genetic diversity of sweet sorghum germplasm in Mexico using AFLP and SSR markers. *Pesqui. Agropecu. Bras.* 47, 1095–1102.

Peng, Y., Li, C., and Fritschi, F. B. (2012). Apoplastic infusion of sucrose into stem internodes during female flowering does not increase grain yield in maize plants grown under nitrogen-limiting conditions. *Physiol. Plant.* doi: 10.1111/j.1399-3054.2012.01711.x [Epub ahead of print].

Philippe, S., Saulnier, L., and Guillon, F. (2006). Arabinoxylan and (1→3),(1→4)-β-glucan deposition in cell walls during wheat endosperm development. *Planta* 224, 449–461. doi: 10.1007/s00425-005-0209-5

Phillips, K. A., Skirpan, A. L., Liu, X., Christensen, A., Slewinski, T. L., Hudson, C., et al. (2011). vanishing tassel2 encodes a grass-specific tryptophan aminotransferase required for vegetative and reproductive development in maize. *Plant Cell* 23, 550–566. doi: 10.1105/tpc.110.075267

Price, M. B., Jelesko, J., and Okumoto, S. (2012). Glutamate receptor homologs in plants: functions and evolutionary origins. *Front. Plant Sci.* 3:235. doi: 10.3389/fpls.2012.00235

Qazi, H. A., Paranjpe, S., and Bhargava, S. (2012). Stem sugar accumulation in sweet sorghum – activity and expression of sucrose metabolizing enzymes and sucrose transporters. *J. Plant Physiol.* 169, 605–613. doi: 10.1016/j.jplph.2012.01.005

Rae, A. L., Perroux, J. M., and Grof, C. P. (2005). Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. *Planta* 220, 817–825. doi: 10.1007/s00425-004-1399-y

Raines, C. A. (2011). Increasing photosynthetic carbon assimilation in C3 plants to improve crop yield: current and future strategies. *Plant Physiol.* 155, 36–42. doi: 10.1104/pp.110.168559

Ramon, M., Rolland, F., Sheen, J., Ramon, M., Rolland, F., and Sheen, J. (2008). Sugar sensing and signaling. *Arabidopsis Book* 6:e0117. doi: 10.1199/tab.0117

Ransom-Hodgkins, W., Vaughn, M., and Bush, D. (2003). Protein phosphorylation plays a key role in sucrose-mediated transcriptional regulation of a phloem-specific proton-sucrose symporter. *Planta* 217, 483–489. doi: 10.1007/s00425-003-1011-x

Reddy, B., Ramesh, S., Reddy, P., Ramaih, B., Salimath, P., and Kachapar, R. (2005). Sweet sorghum – a potential alternate raw material for bio-ethanol and bio-energy. *Int. Sorghum Millets Newslett.* 46, 79–86.

Reeves, P. H., Ellis, C. M., Ploense, S. E., Wu, M.-F., Yadav, V., Tholl, D., et al. (2012). A regulatory network for coordinated flower maturation. *PLoS Genet.* 8:e1002506. doi: 10.1371/journal.pgen.1002506

Reid, J. S. G., Davies, C., and Meier, H. (1977). Endo-β-mannanase, the leguminous aleurone layer and the storage galactomannan in germinating seeds of *Trigonella foenum-graecum* L. *Planta* 133, 219–222. doi: 10.1007/BF00380680

Reid, J. S. G., and Meier, H. (1972). The function of the aleurone layer during galactomannan mobilisation in germinating seeds of fenugreek (*Trigonella foenum-graecum* L.), crimson clover (*Trifolium incarnatum* L.) and lucerne (*Medicago sativa* L.): a correlative biochemical and ultrastructural study. *Planta* 106, 44–60. doi: 10.1007/BF00385472

Rhoades, M., and Carvalho, A. (1944). The function and structure of the parenchyma sheath plastids of the maize leaf. *Bull. Torrey Bot. Club* 71, 335–346. doi: 10.2307/2481307

Ritter, K., Jordan, D., Chapman, S., Godwin, I., Mace, E., and McIntyre, L. C. (2008). Identification of QTL for sugar-related traits in a sweet x grain sorghum (*Sorghum bicolor* L. Moench) recombinant inbred population. *Mol. Breed.* 22, 367–384. doi: 10.1007/s11032-008-9182-6

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57, 675–709. doi: 10.1146/annurev.arplant.57.032905.105441

Rolletschek, H., Koch, K., Wobus, U., and Borisjuk, L. (2005). Positional cues for the starch/lipid balance in maize kernels and resource partitioning to the embryo. *Plant J.* 42, 69–83. doi: 10.1111/j.1365-313X.2005.02352.x

Rolletschek, H., Melkus, G., Grafahrend-Belau, E., Fuchs, J., Heinzel, N., Schreiber, F., et al. (2011). Combined noninvasive imaging and modeling approaches reveal metabolic compartmentation in the barley endosperm. *Plant Cell* 23, 3041–3054. doi: 10.1105/tpc.111.087015

Rooney, W. L., Blumenthal, J., Bean, B., and Mullet, J. E. (2007). Designing sorghum as a dedicated bioenergy feedstock. *Biofuels Bioprod. Biorefin.* 1, 147–157. doi: 10.1002/bbb.15

Rossouw, D., Kossmann, J., Botha, F. C., and Groenewald, J. (2010). Reduced neutral invertase activity in the culm tissues of transgenic sugarcane plants results in a decrease in respiration and sucrose cycling and an increase in the sucrose to hexose ratio. *Funct. Plant Biol.* 37, 22–31. doi: 10.1071/FP08210

Ruan, Y.-L., Jin, Y., Yang, Y.-J., Li, G.-J., and Boyer, J. S. (2010). Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. *Mol. Plant* 3, 942–955. doi: 10.1093/mp/ssq044

Ruan, C.-J., Shao, H.-B., and Teixeira da Silva, J. A. (2012a). A critical review on the improvement of photosynthetic carbon assimilation in C3 plants using genetic engineering. *Crit. Rev. Biotechnol.* 32, 1–21. doi: 10.3109/07388551.2010.533119

Ruan, Y.-L., Patrick, J. W., Bouzayen, M., Osorio, S., and Fernie, A. R. (2012b). Molecular regulation of seed and fruit set. *Trends Plant Sci.* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Sabelli, P. A., and Larkins, B. A. (2009). The development of endosperm in grasses. *Plant Physiol.* 149, 14–26. doi: 10.1104/pp.108.129437

Sakamoto, T., Morinaka, Y., Ohnishi, T., Sunohara, H., Fujioka, S., Ueguchi-Tanaka, M., et al. (2006). Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. *Nat. Biotechnol.* 24, 105–109. doi: 10.1038/nbt1173

Sauer, N. (2007). Molecular physiology of higher plant sucrose transporters. *FEBS Lett.* 581, 2309–2317. doi: 10.1016/j.febslet.2007.03.048

Schafer, W. E., Rohwer, J. M., and Botha, F. C. (2004). Protein-level expression and localization of sucrose synthase in the sugarcane culm. *Physiol. Plant.* 121, 187–195. doi: 10.1111/j.0031-9317.2004.00316.x

Schmalstig, J. G., and Hitz, W. D. (1987). Transport and metabolism of a sucrose analog (1′-fluorosucrose) into *Zea mays* L. endosperm without invertase hydrolysis. *Plant Physiol.* 85, 902–905. doi: 10.1104/pp.85.4.902

Seebauer, J. R., Moose, S. P., Fabbri, B. J., Crossland, L. D., and Below, F. E. (2004). Amino acid metabolism in maize earshoots. Implications for assimilate preconditioning and nitrogen signaling. *Plant Physiol.* 136, 4326–4334. doi: 10.1104/pp.104.043778

Seebauer, J. R., Singletary, G. W., Krumpelman, P. M., Ruffo, M. L., and Below, F. E. (2010). Relationship of source and sink in determining kernel composition of maize. *J. Exp. Bot.* 61, 511–519. doi: 10.1093/jxb/erp324

Setter, T. L., and Parra, R. (2010). Relationship of carbohydrate and abscisic acid levels to kernel set in maize under postpollination water deficit. *Crop Sci.* 50, 980–988. doi: 10.2135/cropsci2009.07.0391

Shannon, J. C. (1972). Movement of 14C-labeled assimilates into kernels of *Zea mays* L. I. Pattern and rate of sugar movement. *Plant Physiol.* 49, 198–202. doi: 10.1104/pp.49.2.198

Shannon, J. C., and Dougherty, C. (1972). Movement of 14C-Labeled assimilates into kernels of *Zea mays* L. II. Invertase activity of the pedicel and placento-chalazal tissues. *Plant Physiol.* 49, 203–206. doi: 10.1104/pp.49.2.203

Sharma, S., Sreenivasulu, N., Harshavardhan, V., Seiler, C., Sharma, S., Khalil, Z., et al. (2010). Delineating the structural, functional and evolutionary relationships of sucrose phosphate synthase gene family II in wheat and related grasses. *BMC Plant Biol.* 10:134. doi: 10.1186/1471-2229-10-134

Shen, B., Allen, W. B., Zheng, P., Li, C., Glassman, K., Ranch, J., et al. (2010). Expression of ZmLEC1 and ZmWRI1 increases seed oil production in maize. *Plant Physiol.* 153, 980–987. doi: 10.1104/pp.110.157537

Shevell, D. E., Kunkel, T., and Chua, N.-H. (2000). Cell wall alterations in the *Arabidopsis* emb30 mutant. *Plant Cell* 12, 2047–2059.

Singletary, G. W., Doehlert, D. C., Wilson, C. M., Muhitch, M. J., and Below, F. E. (1990). Response of enzymes and storage proteins of maize endosperm to nitrogen supply. *Plant Physiol.* 94, 858–864. doi: 10.1104/pp.94.3.858

Slewinski, T. L. (2011). Diverse functional roles of monosaccharide transporters and their homologs in vascular plants: a physiological perspective. *Mol. Plant* 4, 641–662. doi: 10.1093/mp/ssr051

Slewinski, T. L. (2012). Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. *J. Exp. Bot.* 63, 4647–4670. doi: 10.1093/jxb/ers124

Slewinski, T. L., and Braun, D. M. (2010). Current perspectives on the regulation of whole-plant carbohydrate partitioning. *Plant Sci.* 178, 341–349. doi: 10.1016/j.plantsci.2010.01.010

Slewinski, T. L., Garg, A., Johal, G. S., and Braun, D. M. (2010). Maize SUT1 functions in phloem loading. *Plant Signal. Behav.* 5, 687–690. doi: 10.4161/psb.5.6.11575

Slewinski, T. L., Ma, Y., Baker, R. F., Huang, M., Meeley, R., and Braun, D. M. (2008). Determining the role of Tie-dyed1 in starch metabolism: epistasis analysis with a maize ADP-glucose pyrophosphorylase mutant lacking leaf starch. *J. Hered.* 99, 661–666. doi: 10.1093/jhered/esn062

Slewinski, T. L., Meeley, R., and Braun, D. M. (2009). Sucrose transporter1 functions in phloem loading in maize leaves. *J. Exp. Bot.* 60, 881–892. doi: 10.1093/jxb/ern335

Smidansky, E. D., Clancy, M., Meyer, F. D., Lanning, S. P., Blake, N. K., Talbert, L. E., et al. (2002). Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1724–1729. doi: 10.1073/pnas.022635299

Smith, A. M., and Stitt, M. (2007). Coordination of carbon supply and plant growth. *Plant Cell Environ.* 30, 1126–1149. doi: 10.1111/j.1365-3040.2007.01708.x

Tai, P. Y. P., and Miller, J. D. (2002). Germplasm diversity among four sugarcane species for sugar composition. *Crop Sci.* 42, 958–964. doi: 10.2135/cropsci2002.0958

Tang, A.-C., and Boyer, J. S. (2013). Differences in membrane selectivity drive phloem transport to the apoplast from which maize florets develop. *Ann. Bot.* 111, 551–562. doi: 10.1093/aob/mct1012

Tarpley, L., Vietor, D., and Miller, F. (1994). Internodal compartmentation of stem-infused [14C]sucrose in sweet and grain sorghum. *Crop Sci.* 34, 1116–1120. doi: 10.2135/cropsci1994.0011183X003400040052x

Tarpley, L., and Vietor, D. M. (2007). Compartmentation of sucrose during radial transfer in mature sorghum culm. *BMC Plant Biol.* 7:33. doi: 10.1186/1471-2229-7-33

Tarpley, L., Vietor, D. M., and Miller, F. R. (1996). Metabolism of sucrose during storage in intact sorghum stalk. *Int. J. Plant Sci.* 157, 159–163. doi: 10.1086/297334

Tiessen, A., and Padilla-Chacon, D. (2013). Subcellular compartmentation of sugar signalling: links among carbon cellular status, route of sucrolysis, sink-source allocation, and metabolic partitioning. *Front. Plant Sci.* 3:306. doi: 10.3389/fpls.2012.00306

Tomes, D. T., Zinselmeier, C., and Habben, J. E. (2011). *Enhanced Floral Sink Strength and Increased Stability of Seed Set in Plants.* U.S. Patent Application 8,026,411.

Turgeon, R. (2006). Phloem loading: how leaves gain their independence. *BioScience* 56, 15–24. doi: 10.1641/0006-3568(2006)056[0015:PLHLGT]2.0.CO;2

Turgeon, R. (2010). The puzzle of phloem pressure. *Plant Physiol.* 154, 578–581. doi: 10.1104/pp.110.161679

Uribelarrea, M., Below, F. E., and Moose, S. P. (2004). Grain composition and productivity of maize hybrids derived from the illinois protein strains in response to variable nitrogen supply. *Crop Sci.* 44, 1593–1600. doi: 10.2135/cropsci2004.1593

Vargas, W. A., and Salerno, G. L. (2010). The Cinderella story of sucrose hydrolysis: alkaline/neutral invertases, from cyanobacteria to unforeseen roles in plant cytosol and organelles. *Plant Sci.* 178, 1–8. doi: 10.1016/j.plantsci.2009.09.015

Vickers, J. E., Grof, C. P. L., Bonnett, G. D., Jackson, P. A., and Morgan, T. E. (2005). Effects of tissue culture, biolistic transformation, and introduction of PPO and SPS gene constructs on performance of sugarcane clones in the field. *Aust. J. Agric. Res.* 56, 57–68. doi: 10.1071/AR04159

Vilhar, B., Kladnik, A., Blejec, A., Chourey, P. S., and Dermastia, M. (2002). Cytometrical evidence that the loss of seed weight in the miniature1 seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. *Plant Physiol.* 129, 23–30. doi: 10.1104/pp.001826

Waclawovsky, A. J., Sato, P. M., Lembke, C. G., Moore, P. H., and Souza, G. M. (2010). Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. *Plant Biotechnol. J.* 8, 263–276. doi: 10.1111/j.1467-7652.2009.00491.x

Walsh, K. B., Sky, R. C., and Brown, S. M. (2005). The anatomy of the pathway of sucrose unloading within the sugarcane stalk. *Funct. Plant Biol.* 32, 367–374. doi: 10.1071/FP04102

Wang, G., Sun, X., Wang, G., Wang, F., Gao, Q., Sun, X., et al. (2011). Opaque7 encodes an acyl-activating enzyme-like protein that affects storage protein synthesis in maize endosperm. *Genetics* 189, 1281–1295. doi: 10.1534/genetics.111.133967

Wang, Z., Chen, X., Wang, J., Liu, T., Liu, Y., Zhao, L., et al. (2007). Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. *Plant Cell Tissue Organ Cult.* 88, 83–92. doi: 10.1007/s11240-006-9173-4

Weber, H., Borisjuk, L., and Wobus, U. (1997). Sugar import and metabolism during seed development. *Trends Plant Sci.* 2, 169–174. doi: 10.1016/S1360-1385(97)85222-3

Welbaum, G., Meinzer, F., Grayson, R., and Thornham, K. (1992). Evidence for the consequences of a barrier to solute diffusion between the apoplast and vascular bundles in sugarcane stalk tissue. *Funct. Plant Biol.* 19, 611–623.

Welbaum, G. E., and Meinzer, F. C. (1990). Compartmentation of solutes and water in developing sugarcane stalk tissue. *Plant Physiol.* 93, 1147–1153. doi: 10.1104/pp.93.3.1147

Wilson, S., Burton, R., Doblin, M., Stone, B., Newbigin, E., Fincher, G., et al. (2006). Temporal and spatial appearance of wall polysaccharides during cellularization of barley (*Hordeum vulgare*) endosperm. *Planta* 224, 655–667. doi: 10.1007/s00425-006-0244-x

Wiltshire, J. J. J., and Cobb, A. H. (1996). A review of the physiology of potato tuber dormancy. *Ann. Appl. Biol.* 129, 553–569. doi: 10.1111/j.1744-7348.1996.tb05776.x

Wingenter, K., Schulz, A., Wormit, A., Wic, S., Trentmann, O., Hoermiller, I. I., et al. (2010). Increased activity of the vacuolar monosaccharide transporter TMT1 alters cellular sugar partitioning, sugar signaling, and seed yield in *Arabidopsis*. *Plant Physiol.* 154, 665–677. doi: 10.1104/pp.110.162040

Winter, H., and Huber, S. C. (2000). Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. *Crit. Rev. Plant Sci.* 19, 31–68. doi: 10.1016/S0735-2689(01)80002-2

Wu, L., and Birch, R. G. (2007). Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnol. J.* 5, 109–117. doi: 10.1111/j.1467-7652.2006.00224.x

Wu, L., and Birch, R. G. (2010). Physiological basis for enhanced sucrose accumulation in an engineered sugarcane cell line. *Funct. Plant Biol.* 37, 1161–1174. doi: 10.1071/FP10055

Xiong, Y., Li, Q.-B., Kang, B.-H., and Chourey, P. (2011). Discovery of genes expressed in basal endosperm transfer cells in maize using 454 transcriptome sequencing. *Plant Mol. Biol. Rep.* 29, 835–847. doi: 10.1007/s11105-011-0291-8

Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C., and Sheen, J. (2013). Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* 496, 181–186. doi: 10.1038/nature12030

Xu, J., Avigne, W. T., McCarty, D. R., and Koch, K. E. (1996). A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: evidence from a maize invertase gene family. *Plant Cell* 8, 1209–1220.

Yang, B., Sugio, A., and White, F. F. (2006). Os8N3 is a host disease-susceptibility gene for bacterial blight of rice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10503–10508. doi: 10.1073/pnas.0604088103

Yang, X., Guo, Y., Yan, J., Zhang, J., Song, T., Rocheford, T., et al. (2010). Major and minor QTL and epistasis contribute to fatty acid compositions and oil concentration in high-oil maize. *Theor. Appl. Genet.* 120, 665–678. doi: 10.1007/s00122-009-1184-1

Yokoyama, K., Ishijima, S. A., Clowney, L., Koike, H., Aramaki, H., Tanaka, C., et al. (2006). Feast/famine regulatory proteins (FFRPs): *Escherichia coli* Lrp, AsnC and related archaeal transcription factors. *FEMS Microbiol. Rev.* 30, 89–108. doi: 10.1111/j.1574-6976.2005.00005.x

Yuan, M., and Wang, S. (2013). Rice MtN3/saliva family genes and their homologues in cellular organisms. *Mol. Plant* doi: 10.1093/mp/sst1035 [Epub ahead of print].

Zeng, Y., Wu, Y., Avigne, W. T., and Koch, K. E. (1998). Differential regulation of sugar-sensitive sucrose synthases by hypoxia and anoxia indicate complementary transcriptional and posttranscriptional responses. *Plant Physiol.* 116, 1573–1583. doi: 10.1104/pp.116.4.1573

Zhang, W.-H., Zhou, Y., Dibley, K. E., Tyerman, S. D., Furbank, R. T., and Patrick, J. W. (2007). Nutrient loading of developing seeds. *Funct. Plant Biol.* 34, 314–331. doi: 10.1071/FP06271

Zheng, H. J., Wu, A. Z., Zheng, C. C., Wang, Y. F., Cai, R., Shen, X. F., et al. (2009). QTL mapping of maize (*Zea mays*) stay-green traits and their relationship to yield. *Plant Breed.* 128, 54–62. doi: 10.1111/j.1439-0523.2008.01529.x

Zhu, X. G., Long, S. P., and Ort, D. R. (2010). Improving photosynthetic efficiency for greater yield. *Annu. Rev. Plant Biol.* 61, 235–261. doi: 10.1146/annurev-arplant-042809-112206

Zhu, Y. J., Komor, E., and Moore, P. H. (1997). Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiol.* 115, 609–616.

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Regulation of cell division and expansion by sugar and auxin signaling

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Plant growth and development are modulated by concerted actions of a variety of signaling molecules. In recent years, evidence has emerged on the roles of sugar and auxin signals network in diverse aspects of plant growth and development. Here, based on recent progress of genetic analyses and gene expression profiling studies, we summarize the functional similarities, diversities, and their interactions of sugar and auxin signals in regulating two major processes of plant development: cell division and cell expansion. We focus on roles of sugar and auxin signaling in both vegetative and reproductive tissues including developing seed.

Keywords: sugar signaling, auxin signaling, cell division, cell expansion, seed development

INTRODUCTION

Plant growth and development results from a combination of three processes at the cellular level: cell division, cell expansion, and cell differentiation. Cell division or mitosis involves the duplication and separation of complete sets of genetic materials. This genetic information is then selectively transcribed and translated to determine the final shape of cells through cell expansion and differentiation. Cell division and expansion determines cell number and cell size in a mature organ, hence its yield. Over the last decade, genetic analyses and genome-wide gene expression profiling studies have significantly advanced our understanding of the signaling pathways regulating cell proliferation and expansion. In this context, the phytohormone auxin plays prominent roles in regulating both cell proliferation and cell expansion (reviewed in Perrot-Rechenmann, 2010). Rapid advances in the area have helped shed light on the molecular mechanisms regulating auxin homeostasis, transport, and signaling.

Sugars, in addition to their fundamental roles as carbon and energy sources, also act as signaling molecules to regulate gene expression (e.g., Rolland et al., 2002; Hartig and Beck, 2006). The disaccharide sucrose (Suc) is transported through phloem from photosynthetic leaves (source) to sink organs such as root, meristem, flower, developing fruit, and seed. In general, lowered Suc levels stimulate source activities, including photosynthesis, nutrient mobilization, and export. In contrast, higher Suc levels is believed to inhibit photosynthesis in source leaves, but stimulate growth and storage in sink tissues. Phloem unloading of Suc in companion cell and sieve element (CC/SE) complex and its post-phloem transport to recipient sink cells may occur either apoplasmically into cell wall matrix or symplasmically through plasmodesmata. Prior to its use for metabolism and biosynthesis, Suc must be degraded either by invertase (Inv) into glucose (Glc) and fructose (Fru), or by Suc synthase (Sus) into UDP-glucose (UDPG) and Fru. Based on the subcellular localization, Inv is usually divided as cell wall Inv (cw-Inv), vacuolar Inv (vac-Inv), and cytosolic Inv (cyto-Inv; but it may also be expressed in mitochondria, plastids, and nuclei); while Sus may exist as soluble protein in cytoplasm (cyto-Sus) or insoluble isoform bound to the plasma membrane (PM-Sus; Cai et al., 2011) and other intracellular organelles (Ruan, 2012). These sucrolytic enzyme activities are not only critical for primary and secondary metabolism by supplying essential energy and building blocks for plant growth, but also play direct roles in signaling (Ruan, 2012), since Suc and its cleavage hexose are also signaling molecules regulating gene expression (reviewed by Gibson, 2005; Rolland et al., 2006; Eveland and Jackson, 2012). So far, the best studied plant sugar signaling pathway is Glc signaling, mediated by its sensor hexokinase (HXK), which sequentially regulates plant gene expression at transcriptional, translational, and post-translational levels (e.g., Sheen, 1990; Ho et al., 2001; Yanasigawa et al., 2003). In the past few years, a large number of sugar-responsive loci have been identified by genetic approaches, and different sugar-based signaling pathways are deciphered in specific developmental process (reviewed in Smeekens et al., 2010). Mounting evidence also suggests crosstalks among

sugar and various hormone signals, e.g., abscisic acid, ethylene, and cytokinins (Gazzarrini and McCourt, 2001; Hartig and Beck, 2006). Among these, close interactions between sugar and auxin signaling play major roles in various aspects of plant development (Eveland and Jackson, 2012).

This review aims to evaluate recent progress on the regulatory roles of sugar and auxin signaling and their interactions in cell division and expansion from a developmental perspective in both vegetative and reproductive organs.

AUXIN AND SUGAR SIGNALING IN CELL PROLIFERATION

The eukaryotic cell cycle consists of DNA synthesis (S phase) and mitosis (M phase), separated by two gap phases G1 and G2 (**Figure 1**). Mitogenic signals are required for completion of cell cycle, in particular during the transitions from G1 to S and G2 to M phases for proper progression of the cycle; otherwise the cell cycle will be arrested. Some plant cells may skip the M phase under certain developmental processes resulting in endoreduplication and an increase in the degree of ploidy (**Figure 1**; Joubés and Chevalier, 2000). A typical example of cell cycle without mitosis occurs in the syncytial endosperm early in seed development.

As a class of essential plant hormones, auxin has been demonstrated to play a leading role in regulating cell proliferation, especially in the preparation of replication (G1 to S phases). A set of *in vitro* cell culture studies have provided insights into the molecular mechanisms by which auxin regulates cell cycle (**Figure 1**; reviewed in Perrot-Rechenmann, 2010). During G1 phase, auxin was shown to induce the expression of cyclin D gene *cycD3;1* and cyclin-dependent kinase gene *CDKA;1*, and to play important roles in CDKA/CYCD complex assembling. Meanwhile, *KRP1* and *KRP2* transcripts, encoding two of the CDK inhibitors, were reported to be down-regulated after auxin treatment, thereby preserving the phosphorylated CDKA/CYCD complex. Activated CDKA/CYCD complex could provoke phosphorylation of the transcriptional repressor retinoblastoma-related (RBR) protein, and release its target Adenovirus E2 promoter-binding factor A/B (E2FA/B) and dimerization partener A (DPA) complex. Through this post-transcriptional regulation, auxin stabilizes the E2FA/B and DPA complex, which promotes the expression of genes essential for initiating the S phase. Later in the S phase, auxin stimulates the degradation of the F-box SKP2A (S phase kinase-associated protein 2A) by E3 ubiquitin ligase complex SCF (Skp, Cullin, F-box containing complex), indirectly stabilizing the E2 promoter-binding factor C (E2FC) and dimerization parterner B (DPB) complex. The latter represses the expression of S phase genes. Although most data suggest auxin acts as a permissive signal for achieving competence to enter DNA synthesis (G1/S transition), it is also required in the later G2/M transition to complete the mitosis process (**Figure 1**; Urano et al., 2012). However, it is difficult to dissect the effect of auxin on later phases from that at the initial step of the cell cycle. Consistent with its role in expedition of cell cycle process, auxin was also found to promote cell division and delay endoreduplication in developing seeds of legume species *Medicago truncatula* (**Figure 1**; Atif et al., 2012).

Comparing to auxin, our current knowledge about the molecular mechanism of sugar-mediated regulation of cell division is largely derived from studies on cultured suspension cells and mutant seedlings subjected to various sugar treatments. A close correlation was observed between the supply of Glc and the expressions of cyclins, e.g., *cycD2;1*, *D3;2*, *A3;2*, and *B1;2* (**Figure 1**; Riou-Khamlichi et al., 2000; Hartig and Beck, 2006). The D-type cyclins are often mentioned as sensors of external conditions, and associate with cyclin-dependent kinase (e.g., CDKA) to regulate cell cycles (Nieuwland et al., 2007). Meanwhile, A3 and B1 cyclins are required to drive G1/S and G2/M transitions, respectively (Menges et al., 2005). These observations suggest that Glc signaling regulates cell cycle throughout the whole cell cycle process. Noteworthy is that the regulatory effect of Glc on the rate of cell division primarily results from signaling rather than nutrient availability and energy status, as cell proliferating activity positively correlated with endogenous hexose levels, but not their uptake rate (Hartig and Beck, 2006). A recent study in *Arabidopsis* meristematic tissues has shown that Glc signal initiates the G2/M transition by repressing transcription of the negative regulator *TPR-DOMAIN SUPPRESSOR OF STIMPY* (*TSS*), thereby activating the expression of key cell cycle components required for G2/M transition, such as *CYCB1;1* and *CDKB1;1* (**Figure 1**; Skylar et al., 2011). Noteworthy is that Glc feeding is insufficient to trigger mitosis, and auxin is also required for the completion of this process, indicating distinct but coordinated roles of sugar and auxin in G2/M regulation (Skylar et al., 2011).

Other than hexose, downstream components of Suc/Glc signaling factors may also be involved in cell cycle regulation. Trehalose-6-phosphate (T6P) is a newly identified signal molecule which is synthesized from G6P and UDPG by T6P synthase (TPS; Paul et al., 2008). AtTPS1, being the only functional *Arabidopsis* TPS enzyme catalyzes T6P synthesis reaction, was observed interacting with CDKA1 and the kinesin KCA1, while its loss of function mutant *tps1* shown embryo lethal (reviewed in Smeekens et al., 2010). The exact role of T6P in cell cycle remains unclear. However, a downstream target of T6P, Suc non-fermenting1 (Snf1)-related protein kinase (SnRK1), is considered to be a sensor negatively regulating plant growth through crosstalk with cell cycle signaling factors as indicated by studies of its homolog Snf1 in yeast (Francis and Halford, 2006). An inhibition of the catalytic activity of SnRK1 by T6P was revealed both *in vitro* and *in vivo* (see O'Hara et al., 2013). A link between T6P/SnRK1 regulatory system and auxin signaling has also been revealed (discussed later, reviewed in O'Hara et al., 2013), implying a potential molecular mechanism of T6P and SnRK1 signaling in regulating cell cycle. However, SnRK1 signal is currently associated with the response to starvation or low energy status during plant development, as its activity leads to down-regulation of carbon-consuming processes but enhancement of photosynthetic processes, thereby increasing carbon availability (Halford and Hey, 2009; O'Hara et al., 2013). It remains to be determined whether SnRK1 signal plays a role in cells division. Similarly, the perception of cellular energy and nutrient levels typically leads to the activation of the growth promoting target of rapamycin (TOR) protein kinase signaling pathway, which then adjusts cell growth and proliferation accordingly. It has been reported that TOR promotes *Drosophila* female germline stem cells proliferation during G2 phase (LaFever et al., 2010). A single copy of TOR has been found in *Arabidopsis*, the loss of which results

in embryonic lethality (Menand et al., 2002). Whether the TOR complex functions in regulating plant cell division remains to be investigated.

ROLES OF AUXIN AND SUGAR IN CELL EXPANSION

Cell expansion is another important cellular process for plant growth, which is a net result of internal turgor pressure and irreversible cell wall extension. By accumulating sugars, irons and other osmotically active solutes, plant cell generates a lower osmotic potential to attract water flux into the cell, thereby generating a turgor pressure to drive cell expansion. This process also requires the cell wall to be irreversibly stretched through a wall loosening process, followed by deposition of new wall material. The extent and direction of cell expansion is modulated by many factors including cytoskeletons (Li et al., 2001; Pollard and Cooper, 2009).

Auxin was shown to induce rapid cell elongation in stem, coleoptiles, or hypocotyls segments within minutes after auxin treatment (Rayle and Cleland, 1992). Current model of auxin-regulated cell expansion is based on an acid growth theory (**Figure 2**; reviewed in Mockaitis and Estelle, 2008; Perrot-Rechenmann, 2010). An extracellular localized auxin is perceived by the auxin receptor, auxin binding protein 1 (ABP1). An interaction between the ABP1 and some unknown membrane-associated proteins may activate the plasma membrane (PM)-H^+-ATPase, pumping proton into the extracellular space. This lowers the pH in cell wall matrix, activating cell wall loosening proteins such as expansins and xyloglucan endotransglycosylase/hydrolases (XTH), and consequently making the cell wall relaxed for expansion. The PM-H^+-ATPase activity also promotes hyperpolarization of membrane potential which activates voltage-dependent potassium inward channels, thereby contributing to the osmotically driven water uptake for expansion.

In parallel to its role in activating proteins essential for cell expansion at the post-translational level, auxin signal in the nuclei also enhances the transcription of these genes including those encoding PM-ATPase, K^+ channels, expansins, and cell wall remodeling enzymes. Furthermore, auxin promotes exocytosis of vesicles containing new cell wall material (**Figure 2**; reviewed in Perrot-Rechenmann, 2010). In addition, auxin may affect F-actin through ABP1 and its downstream Rho GTPases and their effectors CRIB MOTIF-CONTAINING proteins (RICs), which consequently modulates asymmetric cell expansion (**Figure 2**; Yang and Fu, 2007; Xu et al., 2010).

Different from auxin which regulates cell growth solely through signaling, sugars modulate cell expansion both as important signals and metabolites. The latter serves as osmotically active solutes and substrates for biosynthesis of diverse products including cell wall material required for cell expansion.

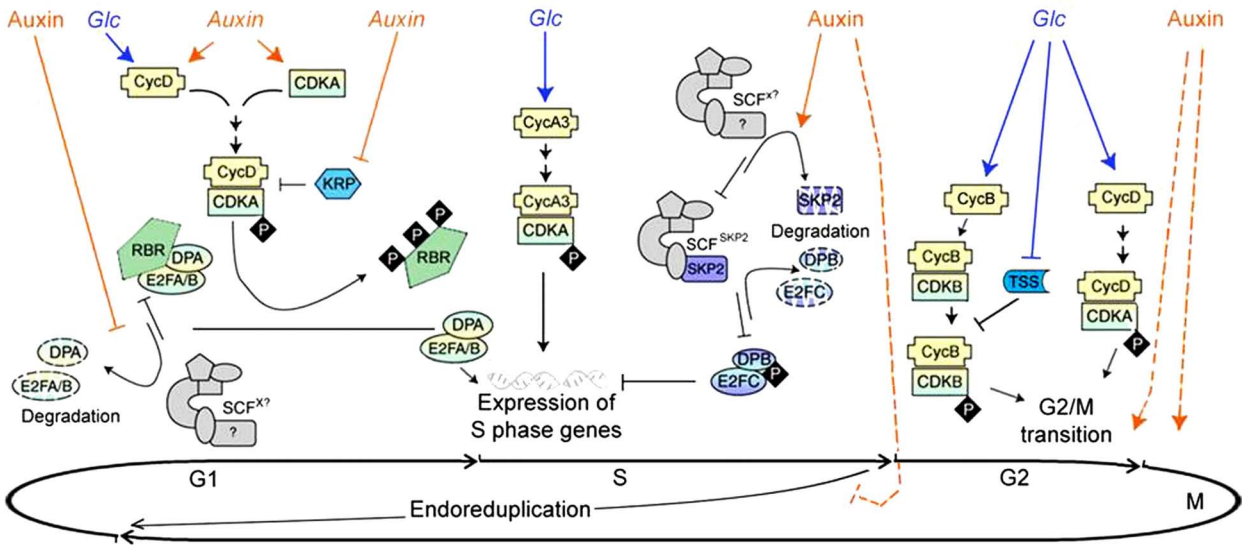

FIGURE 1 | Glucose and auxin signal in cell cycle regulation (modified from Perrot-Rechenmann, 2010, Figure 2). The cell cycle is divided into four phases: DNA replication (S), mitosis (M), and two gap phases (G1 and G2, between M/S and S/M, respectively). Some plant cells may skip the M phase under certain developmental processes, resulting "endoreduplication." Cell cycle starts in G1. During this phase, Glc and auxin signals could induce the expression of *CycD*, while auxin is also able to increase *CDKA* transcription. The CycD/CDKA complex is activated by phosphorylation but can still be blocked by CDK inhibitor KRPs. Auxin was reported to reduce the expression of some *KRPs*. The active CycD/CDKA complex provokes phosphorylation of the transcriptional repressor RBR, and release the transcription regulator E2FA/B and DPA complex. By post-transcriptional regulation, auxin stabilizes the E2FA/B and DPA complex, which promote the expression of genes essential for the beginning of the S phase. The expression of CycA3 could be up-regulated by Glc signal, which is required to drive the cells from G1 to S phase. Auxin was shown to increase the degradation of the F-box SKP2 later in S phase, which indirectly stabilizes E2FC/DPB complex, and represses the S phase genes expression. As cell cycle processes into G2 phase, Glc signal was found to initiate the G2/M transition by repressing *TSS* transcription, and activating the expression of key cell cycle genes, such as *CycB* and *CycD*. Auxin signal is required for the initiation and completion of mitosis, probably though an unknown pathway independent or in parallel to Glc. Auxin is also likely to emit a negative signal to prevent cell from going into endoreduplication hence sustaining cell divisions. Glc and Auxin in italic indicate regulation at transcription level and those in non-italic suggest regulation at protein level.

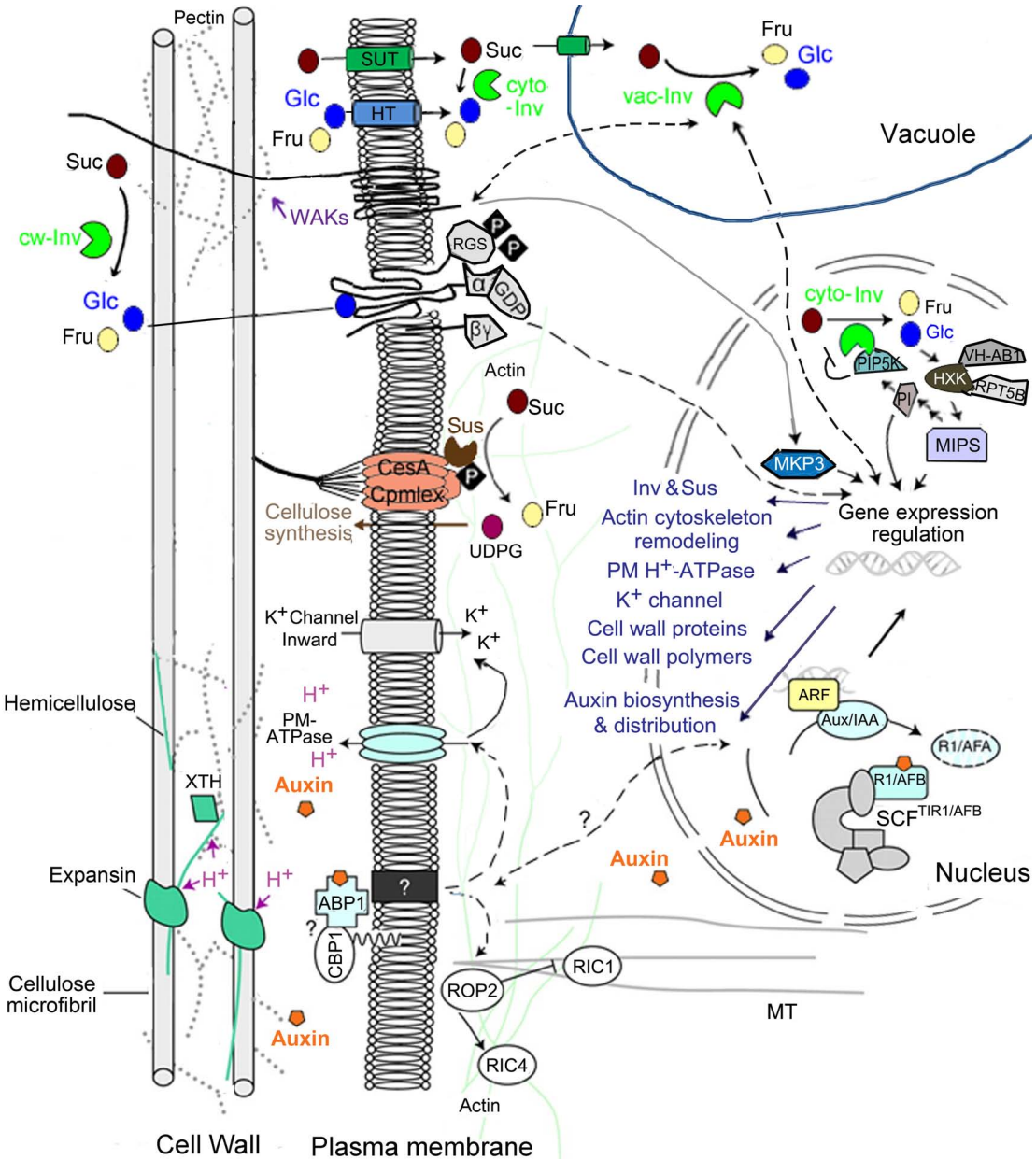

FIGURE 2 | Sugar and auxin signal in regulating sink cell expansion (modified from Perrot-Rechenmann, 2010, Figure 3). Unloaded Suc in sink tissues may enter into recipient cells either apoplasmically through cell wall matrix or symplasmically via plasmodesmatal. In the former case, sucrose could be taken up by sucrose transporter on plasma membrane, or be hydrolyzed by cw-Inv into Glc and Fru, and then be transported into cells by hexose transporter (HT). Apoplasmic Glc could be recognized by RGS1, which transmits extracellular sugar signal into the cell through G-proteins. In cytoplasm, Suc may be hydrolyzed by cyto-Inv or degraded by Sus. In the present of high Suc level, Sus intends to bind actin filaments and form a multi-protein complex bound to plasma membrane, which may facilitate cell expansion by providing UDPG for cellulose/callose biosynthesis. Cytoplasmic Suc could also be transported into nucleus, vacuole, plastid or mitochondrion. In vacuole, Suc could be hydrolyzed by vac-Inv, thus doubling the osmotic contribution of Suc, which has the potential to positively impact on cell turgor. Moreover, vac-Inv could also promote cell expansion via sugar signaling involving WAKs, which subsequently activates MPK3 in nucleus, and induces downstream gene expression for cell wall biosynthesis. Suc hydrolysis in vacuole could also regulates nuclear gene transcription, involving in auxin biosynthesis, distribution and signaling. An *Arabidopsis* cyto-Inv isoform was found in nucleus, where it interacts with and negatively regulated by a phosphatidylinositol monophosphate 5-kinase (AtPIP5K9). Hexoses generated by cyto-Inv could be sensed by a nuclear-localized HXK, producing a Glc signaling complex core combining VHA-B1 and RPT5B, which is sequentially integrated into a signal/metabolites loop modulating cell expansion. Auxin is perceived by the auxin receptor ABP1, which interacts with unknown membrane-associated proteins at the plasma membrane [such as glycosylphosphatidylinositol (GPI)-anchored protein C-terminal peptide-binding protein 1. (CBP1)]. This activates the proton pump ATPase, acidifying extracellular space for optimal function of expansins and XTH and activating K^+ inward rectifying channels, essential for water uptake to sustain cell expansion. Auxin could also enhance these effects by promoting the transcription of these genes. Moreover, auxin is likely to act on actin microfilaments and microtubules via the modulation of ROP GTPases, thereby affecting vesicle delivery to plasma membrane and cell wall matrix.

By hydrolyzing Suc into Glc and Fru, vac-Inv doubles the osmotic contribution of Suc in vacuole, and thus has the potential to positively impact cell turgor in cells accumulating hexoses to high levels, such as elongating cotton fiber (**Figure 2**; Wang et al., 2010). Consistently, high vac-Inv expression or activity has been observed in a range of expanding tissues, for example, maize ovaries (Andersen et al., 2002), grape berry (Davies and Robinson, 1996), carrot taproot (Tang et al., 1999), and a reduction of maize ovary expansion was associated with the decrease of a vac-Inv gene *Ivr2* expression under drought (Andersen et al., 2002).

In agreement with the idea that sugar could also act as signal for cell expansion, vac-Inv has been shown to promote cell expansion in *Arabidopsis* root through an osmotic-independent pathway (Wang et al., 2010). One possible explanation is that vac-Inv may crosstalk with wall-associated kinases (WAKs) through sugar signaling to regulate cell wall extensibility (**Figure 2**). Here, as receptor-like proteins, WAKs are bound to pectin in cell walls, and their activity is required for cell expansion (Anderson et al., 2001; Lally et al., 2001). The observations that vac-Inv activity and *AtvacINV2* transcription were dramatically reduced in *Arabidopsis wak2-1* mutant (Kohorn et al., 2006), and the deletion of *AtvacINV2* (*vin*, Salk_100813) down-regulated the expression of *AtWAK2* (Wang and Ruan, unpublished data), strongly suggest an interplay between vacuole sugar homeostasis and extracellular matrix signaling during cell expansion. Microarray analysis revealed a WAK2-dependent pectin activation of many genes involved in cell wall biosynthesis, which is likely achieved via a downstream mitogen-activated protein kinase AtMAPK3 (**Figure 2**; Kohorn et al., 2009; Kohorn and Kohorn, 2012). This, together with our finding on repression of WAK expression in *vin* mutant also implies that a sugar signal derived from vacuole could play roles in transcription of genes required for cell expansion. Indeed, Suc hydrolysis in vacuole was able to evoke a sugar signal effect on numerous gene expressions, involving auxin biosynthesis, distribution, and auxin signal sensing (Mishra et al., 2009; discussed below). Suggested by the finding that vacuolar H^+-ATPase B1 unit (VHA-B1) interacts with the nuclear-localized AtHXK1 (Cho et al., 2006), a similar protein complex on tonoplast in transmitting vacuolar sugar signals might be expected. However, it remains to be determined as to what is the exact downstream signal pathway of this vacuole sugar signal, and how vac-Inv interacts with cell wall protein WAKs to regulate cell expansion.

Other than vac-Inv, cyto-Inv was also described to play a role in regulating root cell elongation in *Arabidopsis* and rice (Lou et al., 2007; Jia et al., 2008). Interestingly, the *Arabidopsis* cyto-Inv isoform AtCIN1 (At1g35580) was observed in nuclei, where it interacts with a phosphatidylinositol monophosphate 5-kinase (AtPIP5K9), and is negatively regulated by AtPIP5K9 (**Figure 2**; Lou et al., 2007). AtPIP5K is a key enzyme in phosphatidylinositol (PI) signaling pathway, and may directly or indirectly regulate cytoskeleton dynamics via myo-inositol (Lou et al., 2007). Meanwhile, the hexoses derived from nuclear Suc degradation catalyzed by AtCIN1, may be sensed by nuclear-localized AtHXK1 (Cho et al., 2006), and sequentially modulates transcriptions of specific target genes (**Figure 2**). However, the biological functions of the nuclear-localized AtCIN1 and AtHXK1 protein complex are still unclear.

In contrast to Inv, the second Suc-degrading enzyme, Sus, contributes to cell expansion primarily through providing one of the cleavage reaction products, UDPG for cell wall biosynthesis. For example, Sus is highly expressed in cotton cellularizing endosperm cells (Ruan et al., 2008) and seed coat transfer cell undergoing wall in growth (Pugh et al., 2010). Recent studies in *Arabidopsis* and tobacco support a model of PM-Sus-mediated cell wall biosynthesis where PM-Sus binds to actin filaments to initiate the formation of a multi-protein complex and to provide UDPG to callose synthase and cellulose synthase, thus facilitating cell expansion via cellulose/callose biosynthesis (**Figure 2**; Fujii et al., 2010; Cai et al., 2011). Interestingly, a conversion from the cytosolic Sus to its membrane-associated form was detected in the presence of high Suc level, indicating a role of Suc homeostasis in cell wall deposition (Cai et al., 2011). Recently, during *Arabidopsis* seed development, hexose signaling was observed to induce the expression of Sus genes via a HXK-independent pathway (Angeles-Núñez and Tiessen, 2012). Together, these results show that sugars may regulate Sus-mediated cell wall biosynthesis through cellular metabolism as well as signaling network.

Research on sugar signaling has been primarily focused on intracellular processes. It remains virtually unknown how cell senses extracellular signals such as sugars to elicit downstream cellular processes. Ruan et al. (2009) proposed that the apoplasmic Glc generated by cw-Inv could be recognized by a membrane protein, RGS1 (regulator of G-protein signaling 1), which transmits extracellular sugar signal into the cell (**Figure 2**). An insight into the molecular basis of sugar and G-protein signaling crosstalk comes from a recent discovery by Urano et al. (2012) in which Glc could be sensed by AtRGS1 that represses the activity of heterotrimeric G-protein complex. This Glc sensing leads to endocytosis of AtRGS1, hence uncoupling the inhibitory effect of AtRGS1 on AtGPA1 (G-protein α subunit) and consequently activating G-protein signaling (Urano et al., 2012).

Other than G-protein, HXK could also be a potential integrator in regulating cell expansion through Glc signaling, probably via modulating cytoskeleton dynamics (Karve et al., 2008; see **Figure 2**). AtHXK1 was shown to affect F-actin dynamics, thereby influencing the formation and the stability of cytoskeleton-bound polysomes, and the complex membrane trafficking involved in expansion (Balasubramanian et al., 2007). In addition, Cho et al. (2006) suggested a nuclear Glc signaling core formed by nuclear-localized AtHXK1 interacts with VHA-B1 and the 19S regulatory particle of proteasome subunit (RPT5B). *Arabidopsis* VHA-Bs are involved in actin cytoskeleton remodeling via binding and stabilizing F-actin *in vitro* (Ma et al., 2012), thereby potentially affecting cell expansion through modulating actin-guided vesicle trafficking for cell wall synthesis.

Together, the above analyses allow us to formulate a model of cell expansion regulated by sugar and auxin signaling network, covering WAKs, G-protein and nuclear PI signaling, and linking transmission of signals from extracellular environment to different subcellular compartments for a range of cellular processes required for cell enlargement (**Figure 2**).

THE INTERPLAY OF SUGAR AND AUXIN SIGNALING PATHWAYS

As discussed above, sugar and auxin signals play distinctive roles in regulating cell division and expansion. However, the two pathways also interact with each other to regulate plant development and recent studies indeed show that auxin biosynthesis, distribution, and response is regulated directly by sugar signal.

Several biosynthesis pathways of the main auxin, IAA (indole-3-acetic acid) have been postulated in plant. These include the widely distributed IAM (indole-3-acetamide) pathway, the IPA (indole-3-pyruvic acid) pathway, and two possible Brassicaceae species-specific pathways using IAOX (indole-3-acetaldoxime) and IAN (indole-3-acetonitrile) as intermediates (Mano and Nemoto, 2012). All the above pathways synthesize TRP (L-tryptophan) as a precursor (Mano and Nemoto, 2012). Importantly, Glc increases the concentrations of many IAA precursors related to IAM, IAOX, and IAN pathways, as well as three major IAA metabolites and conjugates (oxIAA, IAAsp, and IAGlu), demonstrating a direct positive regulation of Glc in auxin synthesis (Sairanen et al., 2012). *In vivo* evidence consistent with this finding comes from *Arabidopsis* hypocotyls, where an endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation (Lilley et al., 2012).Another important founding by Sairanen et al. (2012) is that the Glc-induced auxin synthesis is negatively regulated by the PHYTOCHROME-INTERACTING FACTOR (PIF) transcription factor family. Considering the up-regulation of PIF gene expression by Suc (Stewart et al., 2011), it is possible that PIF protein act as a switch-off button in Glc induction of auxin biosynthesis at high Suc level or by some specific developmental cues. Further evidence on the positive role of Glc in auxin biosynthesis comes from the maize *miniature1* mutant which lacks the expression of basal kernel-specific cw-Inv (INCW2), leading to miniature seed phenotype. In the mutant seed, the IPA auxin synthesis pathway was down-regulated through decreased expression of a maize *YUCCA* gene (LeClere et al., 2010). *YUCCA* encodes a flavin monooxygenase-like enzyme, which uses IPA as a substrate to produce IAA. This observation suggests that Inv-mediated generation of hexoses is required for auxin biosynthesis in developing seed.

In addition to its role in synthesis, Glc is also implicated in auxin distribution and signaling. To this end, microarray analysis revealed that Glc could regulate as much as 62% of IAA related genes in *Arabidopsis* seedlings, including those encoding auxin receptors TIR1 (transport inhibitor response 1) and ABP1, auxin transporter PIN1, auxin response factors ARF4, ARF8, and a number of genes belonging to auxin induced gene families such as AUX/IAA, GH3, and SAUR (Mishra et al., 2009). Studies using Glc-insensitive mutants also revealed that hexose-mediated sugar signaling partially functions through auxin response (Moore et al., 2003). Genetic studies showed that Suc and Glc stabilize N-MYC DOWN-REGULATED-LIKE1 (NDL1) protein, which interacts with Gβγ dimmers of heterotrimeric G-protein complex, thereby positively regulating auxin transport in root to promote lateral root initiation and emergence (Mudgil et al., 2009). During *Arabidopsis* embryogenesis, nuclear PI signaling was involved in regulating polar transport of auxin effluxer PIN1 via modulating endomembrane structure and trafficking (Luo et al., 2011). Given the importance of hexose substrate for PIs biosynthesis (see previous discussions), this observation implies a possible link of Glc-mediated auxin transport through PI signaling.

Apart from Glc, emerging evidence also indicates connections between the T6P/SnRK1 regulatory system and auxin signaling. For example, microarray analysis in *Arabidopsis* seedlings has demonstrated a down-regulation of *AUX/IAA* genes and auxin receptor gene *TIR1* by elevated T6P level (Paul et al., 2008). SnRK1 has been shown to interact with the SKP1 domain of the SCF complex and the 26S proteasome, possibly through phosphorylating targeted proteins such as AUX/IAA for degradation in SCF–TIR1 complex (Farras et al., 2001).

Most of the studies described above on sugar and auxin signaling were conducted in cell culture systems and vegetative tissues such as developing roots, hypocotyls, leaves, and young seedlings. Reproductive organs are equally, or even more complex than, vegetative tissues in response to sugar and hormonal signals (Ruan et al., 2012). We discussed this issue below by focusing on recent advance obtained from developing seed.

ROLES OF SUGAR AND AUXIN SIGNALING IN SEED DEVELOPMENT

Angiosperm seeds originate from double fertilization, which give birth to the diploid embryo and the triploid endosperm, wrapped by the maternal tissue known as seed coat. Seed formation proceeds by a phase of cell division, which represents a crucial period for seed set, highly sensitive to biotic and abiotic stress, and impacting significantly on seed yield potential (Ruan et al., 2012).

During seed development, the endosperm mother cell initially undergoes nuclear divisions without cell wall formation to generate syncytial endosperm soon after fertilization (Olsen, 2004). Endosperm nuclear division occurs earlier and faster than the embryo cell proliferation (Bate et al., 2004; Nowack et al., 2007). This sequential endosperm and embryo proliferation are tightly coupled to different regulatory mechanisms, for example, sugar signaling. Recent findings on the asymmetric spatial expression of cw-Inv gene *GhCWIN1* in cotton embryo sac, implied a control of the sequential development of endosperm and embryo by Inv-mediated sugar signaling. This may be achieved through establishing a spatial gradient of Glc concentration being higher in the endosperm region than that in the embryo region, thus favoring endosperm nuclear division over embryo cell proliferation during the seed set phase (Wang and Ruan, 2012). Consistently, a cw-Inv inhibitor was localized at the boundary between the endosperm and embryo in developing maize seed (Bate et al., 2004), which could help to minimize hexose flow to young embryo, to ensure nuclear division in endosperm but a quiescent status in embryo at this stage.

Later, embryo develops starting from the acquisition of zygote polarity and elongation, follows by a serial of cell division. Soon after the asymmetric zygote division, the separated cells quickly establish an apical–basal axis of polarity, then the differentiation of an epidermis and the formation of the shoot and root meristem during the next rounds of cell division (Dumas and Rogowsky, 2008). Auxin plays a prominent role in regulating these pattern formations in a cell type-dependent manner (Rademacher et al., 2012). The molecular mechanisms of auxin action in early

embryogenesis have been reviewed (e.g., Möller and Weijers, 2009; Lau et al., 2012), and will not be discussed here. Comparing to auxin, it remains unknown whether sugar signaling affects embryo pattern formation. However, significant correlations between Glc concentration and CWIN expression and early embryo (pre-heart stage) mitotic activity have been revealed in many species, such as *Arabidopsis*, faba bean, and cotton (Weber et al., 1996; Morley-Smith et al., 2008; Wang and Ruan, 2012), indicating Glc as a signal to stimulate cytokinesis during embryogenesis. Apart from Glc, T6P signaling may also affect mitosis activity in early embryo, as *Arabidopsis TPS1*-deficient mutant (*tps1*) shown slower embryo development compare to that of wild-type (Gómez et al., 2010). However, *tps1* embryos are eventually stopped at torpedo stage, implying T6P signal may not essential for rapid cell division in early development, but indispensable for the transition into late stage of embryo development (Gómez et al., 2010).

As embryo develops to heart-torpedo stages, cells undergo rapid expansion, and gradually start to accumulate storage materials. As discussed before, cell expansion could be regulated by the concerted actions of sugar and auxin signaling network. Many studies have shown the impacts of sugar and auxin on seed size (e.g., Cheng and Chourey, 1999; Andersen et al., 2002; Schruff et al., 2006). However, most of the observations are not able to differentiate signaling roles of sugar from its potential nutrient or osmotic effect. The contribution of auxin signal to seed size is largely due to its roles in cell division but not expansion. There is little direct evidence about the role of sugar and auxin signaling in cell enlargement so far, probably due to, in part, the complexity of cell types in seed.

By contrast, many studies have demonstrated that the switch from seed expansion to storage phase is strongly affected by sugar signals. A general finding is that the transition from cell division and expansion to storage activities in seed is usually associated with a decrease in Inv expression and activity and an increase in Sus activity, and an increased ratio of Suc to Glc. Using high-resolution histographical mapping, a clear correlation was observed between decreased Glc concentration and reduced mitotic activity in legume embryo (Borisjuk et al., 1998), largely due to the decrease of cw-Inv genes expression (Weber et al., 1995). In cytoplasm, an increase of Sus activity leads a shift in carbon use toward starch synthesis in plastid. For example, highest expression of *Sus* gene temporally matches with rapid starch filling in rice grain (Wang et al., 1999). In correlation to Suc level, T6P synthesis could be induced, which may then result in an increased expression and activity of the ADP-glucose pyrophosphorylase (AGPase), as well as the redox activation of AGPase, essential for starch accumulation in the plastid (O'Hara et al., 2013).

Clearly, much has been learned about the complexity of sugar and auxin network in regulating plant cell division and expansion. Further research on dissecting the complex network of sugar and auxin signaling pathways in specific plant tissues under particular growth stages or environment conditions, will not only contribute to a better understanding of the regulatory systems underpinning complex developmental processes, but could also have great importance in crop improvement through designing innovative approaches to optimize plant performance for yield and stress tolerance.

REFERENCES

Andersen, M. N., Asch, F. A., Wu, Y., Jensen, C. R., Næsted, H., Mogensen, V. O., et al. (2002). Soluble invertase expression is an early target of stress during the critical, abortion sensitive phase of young ovary development in maize. *Plant Physiol.* 130, 591–604. doi: 10.1104/pp.005637

Anderson, C. M., Wagner, T. A., Perret, M., He, Z. H., He, D., and Kohorn, B. D. (2001). WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. *Plant Mol. Biol.* 47, 197–206. doi: 10.1023/A:1010691701578

Angeles-Núñez, J. G., and Tiessen, A. (2012). Regulation of AtSUS2 and AtSUS3 by glucose and the transcription factor LEC2 in different tissues and at different stages of *Arabidopsis* seed development. *Plant Mol. Biol.* 78, 377–392. doi: 10.1007/s11103-011-9871-0

Atif, R. M., Boulisset, F., Conreux, C., Thompson, R., and Ochatt, S. J. (2012). In vitro auxin treatment promotes cell division and delays endoreduplication in developing seeds of the model legume species *Medicago truncatula*. *Physiol. Plant.* doi: 10.1111/j.1399-3054.2012.01719.x [Epub ahead of print].

Balasubramanian, R., Karve, A., Kandasamy, M., Meagher, R. B., and Moore, B. D. (2007). A role for F-actin in hexokinase-mediated glucose signaling. *Plant Physiol.* 145, 1423–1434. doi: 10.1104/pp.107.108704

Bate, N. J., Niu, X., Wang, Y., Reimann, K. S., and Helentharis, T. G. (2004). An invertase inhibitor from maize localizes to the embryo surrounding region during early kernel development. *Plant Physiol.* 134, 246–154. doi: 10.1104/pp.103.027466

Borisjuk, L., Walenta, S., Weber, H., Mueller-Klieser, W., and Wobus, U. (1998). High-resolution histographical mapping of glucose concentrations in developing cotyledons of *Vicia faba* in relation to mitotic activity and storage processes: glucose as a possible developmental trigger. *Plant J.* 15, 583–591. doi: 10.1046/j.1365-313X.1998.00214.x

Cai, G., Faleri, C., Del Casino, C., Emons, A. M., and Cresti, M. (2011). Distribution of callose synthase, cellulose synthase, and sucrose synthase in tobacco pollen tube is controlled in dissimilar ways by actin filaments and microtubules. *Plant Physiol.* 155, 1169–1190. doi: 10.1104/pp.110.171371

Cheng, W. H., and Chourey, P. S. (1999). Genetic evidence that invertase mediated released of hexoses is critical for appropriate carbon partitioning and normal seed development in maize. *Theor. Appl. Genet.* 98, 485–495. doi: 10.1007/s001220051096

Cho, Y. H., Yoo, S. D., and Sheen, J. (2006). Regulatory functions of nuclear hexokinase 1 complex in glucose signaling. *Cell* 127, 579–589. doi: 10.1016/j.cell.2006.09.028

Davies, C., and Robinson, S. P. (1996). Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. *Plant Physiol.* 111, 275–283. doi: 10.1104/pp.111.1.275

Dumas, C., and Rogowsky, P. (2008). Fertilization and early seed formation. *C.R. Biol.* 331, 715–725. doi: 10.1016/j.crvi.2008.07.013

Eveland, A. L., and Jackson, D. P. (2012). Sugar, signaling, and plant development. *J. Exp. Bot.* 63, 3367–3377. doi: 10.1093/jxb/err379

Farras, R., Ferrando, A., Jasik, J., Kleinow, T., Okresz, L., Tiburcio, A., et al. (2001). SKP1–SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. *EMBO J.* 20, 2742–2756. doi: 10.1093/emboj/20.11.2742

Francis, D., and Halford, G. (2006). Nutrient sensing in plant meristems. *Plant Mol. Biol.* 60, 981–993. doi: 10.1007/s11103-005-5749-3

Fujii, S., Hayashi, T., and Mizuno, K. (2010). Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant Cell Physiol.* 51, 294–301. doi: 10.1093/pcp/pcp190

Gazzarrini, S., and McCourt, P. (2001). Genetic interaction between ABA, ethylene and sugar signaling pathways. *Curr. Opin. Plant Biol.* 4, 387–391. doi: 10.1016/S1369-5266(00)00190-4

Gibson, S. I. (2005). Control of plant development and gene expression by sugar signaling. *Curr. Opin. Plant Biol.* 8, 93–102. doi: 10.1016/j.pbi.2004.11.003

Gómez, L. D., Gilday, A., Feil, R., Lunn, J. E., and Graham, I. A. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for

embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. *Plant J.* 64, 1–13. doi: 10.1111/j.1365-313X.2010.04312.x.

Halford, N. G., and Hey, S. J. (2009). Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signaling in plants. *Biochem. J.* 419, 247–259. doi: 10.1042/BJ20082408

Hartig, K., and Beck, E. (2006). Crosstalk between auxin, cytokinins, and sugars in the plant cell cycle. *Plant Biol.* 8, 389–396. doi: 10.1055/s-2006-923797

Ho, S. L., Chao, Y. C., Tong, W. F., and Yu, S. M. (2001). Sugar coordinately and differentially regulates growth and stress-related gene expression via a complex signal transduction network and multiple control mechanisms. *Plant Physiol.* 125, 877–890. doi: 10.1104/pp.125.2.877

Jia, L., Zhang, B., Mao, C., Li, J., Wu, Y., Wu, P., et al. (2008). OsCYT-INV1 for alkaline/neutral invertase is involved in root cell development and reproductivity in rice (*Oryza sativa* L.). *Planta* 228, 51–59. doi: 10.1007/s00425-008-0718-0

Joubés, J., and Chevalier, C. (2000). Endoreduplication in higher plants. *Plant Mol. Biol.* 43, 735–745. doi: 10.1023/A:1006446417196

Karve, A., Rauh, B. L., Xia, X., Kandasamy, M., Meagher, R. B., Sheen, J., et al. (2008). Expression and evolutionary features of the hexokinase gene family in *Arabidopsis*. *Planta* 228, 411–425. doi: 10.1007/s00425-008-0746-9

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., et al. (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60, 974–982. doi: 10.1111/j.1365-313X.2009.04016.x

Kohorn, B. D., Kobayashi, M., Johansen, S., Riese, J., Huang, L. F., Koch, K., et al. (2006). An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* 46, 307–316. doi: 10.1111/j.1365-313X.2006.02695.x

Kohorn, B. D., and Kohorn, S. L. (2012). The cell wall-associated kinases, WAKs, as pectin receptors. *Front. Plant Sci.* 3:88. doi: 10.3389/fpls.2012.00088

LaFever, L., Feoktistov, A., Hsu, H. J., and Drummond-Barbosa, D. (2010). Specific roles of target of rapamycin in the control of stem cells and their progeny in the *Drosophila* ovary. *Development* 137, 2117–2126. doi: 10.1242/dev.050351

Lally, D., Ingmire, P., Tong, H. Y., and He, Z. H. (2001). Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. *Plant Cell* 13, 1317–1331.

Lau, S., Slane, D., Herud, O., Kong, J., and Jürgens, G. (2012). Early embryogenesis in flowering plants: setting up the basic body pattern. *Annu. Rev. Plant Biol.* 63, 6.1–6.24. doi: 10.1146/annurev-arplant-042811-105507

LeClere, S., Schmelz, E. A., and Chourey, P. S. (2010). Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. *Plant Physiol.* 153, 306–318. doi: 10.1104/pp.110.155226

Li, Y., Zee, S. Y., Liu, Y. M., Huang, B. Q., and Yen, L. F. (2001). Circular F-actin bundles and a G-actin gradient in pollen and pollen tubes of *Lilium davidii*. *Planta* 213, 722–730. doi: 10.1007/s004250100543

Lilley, J. L., Gee, C. W., Sairanen, I., Ljung, K., and Nembhauser, J. L. (2012). An endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation. *Plant Physiol.* 160, 2261–2270. doi: 10.1104/pp.112.205575

Lou, Y., Gou, J. Y., and Xue, H. W. (2007). PIP5K9, an *Arabidopsis* phosphatidylinositol monophosphate kinase, interacts with a cytoplast invertase to negatively regulate sugar-mediated root growth. *Plant Cell* 19, 163–181. doi: 10.1105/tpc.106.045658

Luo, Y., Qin, G., Zhang, J., Liang, Y., Song, Y., Zhao, M., et al. (2011). D-myo-inositol-3-phosphate affects phosphatidylinositol-mediated endomembrane function in *Arabidopsis* and is essential for auxin-regulated embryogenesis. *Plant Cell* 23, 1352–1372. doi: 10.1105/tpc.111.083337

Ma, B., Qian, D., Nan, Q., Tan, C., An, L., and Xiang, Y. (2012). *Arabidopsis* vacuolar H^+-ATPase (V-ATPase) B subunits are involved in actin cytoskeleton remodeling via binding to, bundling, and stabilizing F-actin. *J. Biol. Chem.* 287, 19008–19017. doi: 10.1074/jbc.M111.281873

Mano, Y., and Nemoto, K. (2012). The pathway of auxin biosynthesis in plants. *J. Exp. Bot.* 63, 2853–2872. doi: 10.1093/jxb/ers091

Menand, B., Desnos, T., Nussaume, L., Berger, F., Bouchez, D., Meyer, C., et al. (2002). Expression and disruption of the *Arabidopsis* TOR (target of rapamycin) gene. *Proc. Natl. Acad. Sci. U.S.A.* 99, 6422–6427. doi: 10.1073/pnas.092141899

Menges, M., de Jager, S. M., Gruissem, W., and Murray, J. A. (2005). Global analysis of the core cell cycle regulators of *Arabidopsis* identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. *Plant J.* 41, 546–566. doi: 10.1111/j.1365-313X.2004.02319.x

Mishra, B. S., Singh, M., Aggrawal, P., and Laxmi, A. (2009). Glucose and auxin signaling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. *PLoS ONE* 4:e4502. doi: 10.1371/ journal.pone.0004502

Mockaitis, K., and Estelle, M. (2008). Auxin receptors and plant development: a new signaling paradigm. *Annu. Rev. Cell Dev. Biol.* 24, 55–80. doi: 10.1146/annurev.cellbio.23.090506.123214

Möller, B., and Weijers, D. (2009). Auxin control of embryo patterning. *Cold Spring Harb. Perspect. Biol.* 1, a001545. doi: 10.1101/cshperspect.a001545

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W. H., Liu, Y. X., et al. (2003). Roles of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* 300, 332–336. doi: 10.1126/science.1080585

Morley-Smith, E. R., Pike, M. J., Findlay, K., Kockenberger, W., Hill, L. M., Smith, A. M., et al. (2008). The transport of sugars to developing embryos is not via the bulk endosperm in oilseed rape seeds. *Plant Physiol.* 147, 2121–2130. doi: 10.1104/pp.108.124644

Mudgil, Y., Uhrig, J. F., Zhou, J., Temple, B., Jiang, K., and Jones, A. M. (2009). *Arabidopsis* N-MYC DOWNREGULATED-LIKE1, a positive regulator of auxin transport in a G protein-mediated pathway. *Plant Cell* 21, 3591–3609. doi: 10.1105/tpc.109.065557

Nieuwland, J., Menges, M., and Murray, J. A. H. (2007). "The plant cyclins," in *Cell Cycle Control and Plant Development*, ed. D. Inze (Oxford: Blackwell Publishing), 31–61. doi: 10.1002/9780470988923.ch2

Nowack, M. K., Shirzadi, R., Dissmeyer, N., Dolf, A., Endl, E., Grini, P. E., et al. (2007). Bypassing genomic imprinting allows seed development. *Nature* 447, 312–315. doi: 10.1038/nature05770

O'Hara, L. E., Paul, M. J., and Wingler, A. (2013). How do sugars regulate plant growth and development? New insight into the role of trehalose-6-phosphate. *Mol. Plant* 6, 261–274. doi: 10.1093/mp/sss120

Olsen, O.-A. (2004). Nuclear endosperm development in Cereals and *Arabidopsis thaliana*. *Plant Cell* 16(Suppl. 1), S214–227. doi: 10.1105/tpc.017111

Paul, M. J., Primavesi, L. F., Jhurreea, D., and Zhang, Y. (2008). Trehalose metabolism and signaling. *Annu. Rev. Plant Biol.* 59, 417–441. doi: 10.1146/annurev.arplant.59.032607.092945

Perrot-Rechenmann, C. (2010). Cellular responses to auxin: division versus expansion. *Cold Spring Harb. Perspect. Biol.* 2, a001446. doi: 10.1101/cshperspect.a001446

Pollard, T. D., and Cooper, J. A. (2009). Actin, a central player in cell shape and movement. *Science* 326, 1208–1212. doi: 10.1126/science.1175862

Pugh, D. A., Offler, C. E., Talbot, M. J., and Ruan, Y. L. (2010). Evidence for the role of transfer cells in the evolutionary increase in seed and fiber biomass yield in cotton. *Mol. Plant* 3, 1075–1086. doi: 10.1093/mp/ssq054

Rademacher, E. H., Lokerse, A. S., Schlereth, A., Llavata-Peris, C. I., Bayer, M., Kientz, M., et al. (2012). Different auxin response machineries control distinct cell fates in the early plant embryo. *Dev. Cell* 22, 211–222. doi: 10.1016/ j.devcel.2011.10.026

Rayle, D. L., and Cleland, R. E. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 99, 1271–1274. doi: 10.1104/pp.99.4.1271

Riou-Khamlichi, C., Menges, M., Healy, J. M., and Murray, J. A. (2000). Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. *Mol. Cell. Biol.* 20, 4513–4521. doi: 10.1128/MCB.20.13.4513-4521.2000

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plant: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57, 675–709. doi: 10.1146/annurev.arplant.57.032905.105441

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. *Plant Cell* 14, S185–S205. doi: 10.1105/tpc.010455

Ruan, Y.-L. (2012). Signaling roles of sucrose metabolism in plant development Mol. *Plant* 5, 763–765. doi: 10.1093/mp/sss046

Ruan, Y.-L., Jin, Y., and Huang, J. (2009). Capping invertase activity by its inhibitor: roles and implications in sugar signaling, carbon allocation, senescence and evolution. *Plant Signal. Behav.* 4, 983–986. doi: 10.4161/psb.4.10.9667

Ruan, Y.-L., Llewellyn, D. J., Liu, Q., Xu, S. M., Wu, L. M., Wang, L., et al. (2008). Expression of sucrose synthase in the developing endosperm is essential for early seed development in cotton. *Funct. Plant Biol.* 35, 382–393. doi: 10.1071/FP08017

Ruan, Y. L., Patrick, J. W., Bouzayen, M., Osorio, S., and Fernie, A. R. (2012). Molecular regulation of seed and fruit set. *Trends Plant Sci.* 17, 656–665. doi: 10.1016/j.tplants.2012.06.005

Sairanen, I., Novák, O., Pencík, A., Ikeda, Y., Jones, B., Sandberg, G., et al. (2012). Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in *Arabidopsis*. *Plant Cell* 24, 4907–4916. doi: 10.1105/tpc.112.104794

Schruff, M. C., Spielman, M., Tiwari, S., Adams, S., Fenby, N., and Scott, R. J. (2006). The AUXIN RESPONSE FACTOR 2 gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. *Development* 133, 251–261. doi: 10.1242/dev.02194

Sheen, J. (1990). Metabolic repression of transcription in higher plants. *Plant Cell* 2, 1027–1038. doi: 10.1105/tpc.2.10.1027

Skylar, A., Sung, F., Hong, F., Chory, J., and Wu, X. (2011). Metabolic sugar signal promotes *Arabidopsis* meristematic proliferation via G2. *Dev. Biol.* 351, 82–89. doi: 10.1016/j.ydbio.2010.12.019

Smeekens, S., Ma, J., and Rolland, F. (2010). Sugar signals and molecular networks controlling plant growth. *Curr. Opin. Plant Biol.* 13, 177–191. doi: 10.1016/j.pbi.2009.12.002

Stewart, J. L., Maloof, J. N., and Nemhauser, J. L. (2011). PIF genes mediate the effect of sucrose on seedling growth dynamics. *PLoS Genet.* 8:e1002594. doi: 10.1371/journal.pone.0019894

Tang, G. Q., Luscher, M., and Sturm, A. (1999). Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. *Plant Cell* 11, 177–189. doi: 10.1105/tpc.11.2.177

Urano, D., Phan, N., Jones, J. C., Yang, J., Huang, J. R., Grigston, J., et al. (2012). Endocytosis of the seven-transmembrane RGS1 protein activates G-protein-coupled signalling in *Arabidopsis*. *Nat. Cell Biol.* 14, 1079–1088. doi: 10.1038/ncb2568

Wang, A.-Y., Kao, M.-H., Yang, W.-H., Sayion, Y., Liu, L.-F., Lee, P.-D., et al. (1999). Differentially and developmentally regulated expression of three rice sucrose synthase genes. *Plant Cell Physiol.* 40, 800–807. doi: 10.1093/oxfordjournals.pcp.a029608

Wang, L., Li, X. R., Lian, H., Ni, D. A., He, Y. K., Chen, X. Y., et al. (2010). Evidence that high activity of vacuolar invertase is required for cotton fiber and *Arabidopsis* root elongation through osmotic dependent and independent pathway, respectively. *Plant Physiol.* 154, 744–756. doi: 10.1104/pp.110.162487

Wang, L., and Ruan, Y.-L. (2012). New insights into roles of cell wall invertase in early seed development revealed by comprehensive spatial and temporal expression patterns of GhCWIN1 in cotton. *Plant Physiol.* 160, 777–787. doi: 10.1104/pp.112.203893

Weber, H., Borisjuk, L., Heim, U., Buchner, P., and Wobus, U. (1995). Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. *Plant Cell* 7, 1835–1846. doi: 10.1105/tpc.7.11.1835

Weber, H., Borisjuk, L., and Wobus, U. (1996). Controlling seed development and seed size in *Vicia faba*: a role for seed coat-associated invertases and carbohydrate state. *Plant J.* 10, 823–834. doi: 10.1046/j.1365-313X.1996.10050823.x

Xu, T., Wen, M., Nagawa, S., Fu, Y., Chen, J.-G., Wu, M.-J., et al. (2010). Cell surface- and Rho GTPase-based auxin signaling controls cellular interdigitation in *Arabidopsis*. *Cell* 143, 99–110. doi: 10.1016/j.cell.2010.09.003

Yanasigawa, S., Yoo, S. D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signaling in plants. *Nature* 425, 521–525. doi: 10.1038/nature01984

Yang, Z., and Fu, Y. (2007). ROP/RAC GTPase signaling. *Curr. Opin. Plant Biol.* 10, 490–494. doi: 10.1016/j.pbi.2007.07.005

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Disruption of a rice gene for α-glucan water dikinase, *OsGWD1*, leads to hyperaccumulation of starch in leaves but exhibits limited effects on growth

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To identify potential regulators of photoassimilate partitioning, we screened for rice mutant plants that accumulate high levels of starch in the leaf blades, and a mutant line leaf starch excess 1 (LSE1) was obtained and characterized. The starch content in the leaf blades of LSE1 was more than 10-fold higher than that in wild-type plants throughout the day, while the sucrose content was unaffected. The gene responsible for the LSE1 phenotype was identified by gene mapping to be a gene encoding α-glucan water dikinase, *OsGWD1* (Os06g0498400), and a 3.4-kb deletion of the gene was found in the mutant plant. Despite the hyperaccumulation of starch in their leaf blades, LSE1 plants exhibited no significant change in vegetative growth, presenting a clear contrast to the reported mutants of *Arabidopsis thaliana* and *Lotus japonicus* in which disruption of the genes for α-glucan water dikinase leads to marked inhibition of vegetative growth. In reproductive growth, however, LSE1 exhibited fewer panicles per plant, lower percentage of ripened grains and smaller grains; consequently, the grain yield was lower in LSE1 plants than in wild-type plants by 20∼40%. Collectively, although α-glucan water dikinase was suggested to have universal importance in leaf starch degradation in higher plants, the physiological priority of leaf starch in photoassimilate allocation may vary among plant species.

Keywords: assimilate partitioning, gene disruption mutant, α-glucan water dikinase, leaf starch, rice

INTRODUCTION

Assimilate partitioning has long been recognized as a target for crop improvement because it can limit the yield potential of the crop plants. Despite the great importance of rice as a major crop, most of the mechanisms of photoassimilate allocation in rice are yet to be elucidated. Utilizing mutants that exhibit phenotypes relevant to a particular biological phenomenon can be a powerful approach to uncovering potential regulators of that phenomenon. In the source leaves of higher plants, both starch and sucrose are the major primary products of photosynthesis; therefore any anomaly in the accumulation of the two compounds may arise from mutations in the genes involved in assimilate allocation. In particular, the accumulation of an extraordinarily high level of leaf starch is easily recognizable by iodine staining, and thus has been repeatedly reported as the "starch excess" phenotype. Starch excess in the leaves is often observed when any inhibition occurs (1) in the export of photoassimilate from source leaves or (2) in starch metabolism in the leaves. The former case includes disruption or suppression of the phloem-loading sucrose transporter (Bürkle et al., 1998; Gottwald et al., 2000) and blockade of the route of assimilate transport (Russin et al., 1996). Cold-girdling of the stem is also known to induce the starch excess phenotype through the impairment of photoassimilate transport (e.g., Krapp et al., 1993). As an instance of the latter case, the *Arabidopsis thaliana* Starch EXcess 1 (SEX1) mutant (Caspar et al., 1991) is the most well-known because of its significant contribution to the current understanding of leaf starch metabolism, i.e., α-glucan water dikinase (GWD, EC 2.7.9.4)-mediated degradation. Disruption of the plastid-localized maltose translocator (Niittylä et al., 2004) is also known to result in starch excess in the leaf, as well as some other mutations of starch degradation-related enzymes (Critchley et al., 2001). As is clear from the above described examples, the study of mutant plants exhibiting the starch excess phenotype in their leaves is a good approach to acquiring a better understanding of the mechanisms and regulation of photoassimilate allocation.

Thus far, no rice mutant that accumulates a high level of starch in the leaf blades has been reported. However, in our preliminary experiments, we found that pronounced starch accumulation occurred in detached leaf blades when sucrose was fed through their cut ends. This finding suggested that a rice mutant exhibiting the starch excess phenotype could exist and that it might be caused by a disorder in the mechanism controlling photoassimilate partitioning. We therefore decided to screen for mutant lines accumulating high levels of starch in their leaf blades. Here we report the isolation and characterization of the first leaf starch excess mutant of rice.

MATERIALS AND METHODS

SCREENING OF THE LEAF STARCH EXCESS MUTANTS

In this study, we used a collection of rice mutant lines induced by the insertion of the endogenous retrotransposon Tos17 (Miyao et al., 2003). To screen for mutants displaying the leaf starch excess (LSE) phenotype, 20 seeds of the M2 or M3 generation of Tos17 mutant lines were grown to the fourth leaf stage in the greenhouse. The terrestrial parts were sampled in the morning and leaf starch was visualized by iodine staining.

GENE MAPPING

Mapping populations were obtained from crosses between mutants with the *japonica* background and an *indica*/*japonica* crossbred rice cultivar "Takanari." In the F2 generation, mutant plants were selected by iodine staining of the terrestrial parts. DNA was extracted according to the method reported by Wang et al. (1993) from the leaf segments of plants showing the mutant phenotype. Gene mapping was carried out using micro-satellite SSR markers (McCouch et al., 2002).

ANALYSIS OF THE GENE STRUCTURE

Genomic DNA was extracted from green leaves by using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA) and was used as the template for polymerase chain reaction (PCR) with PrimeStar GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions. The nucleotide sequences of the PCR primers are listed in **Table 1**. For the analysis of the transcript structures, total RNA was extracted from the leaf blades and reverse-transcribed as below, and the resultant cDNA was used as the template for PCR with ExTaq DNA polymerase (Takara Bio Inc.).

GENERATION OF TRANSGENIC RICE PLANTS

For complementation analysis a 13.2-kb genomic DNA fragment containing the entire open reading frame (ORF) along with 2.2-kb sequence upstream of the putative translation start point of *OsGWD1* (Os06g0498400) was amplified by PCR using the primer pair, GWD1-PL2/R14 (**Table 1**), genomic DNA of the rice cultivar Nipponbare (as the template), and PrimeStar GXL DNA polymerase (Takara Bio Inc.). The DNA fragment was introduced into a transformation vector, pZH2B (Kuroda et al., 2010). For gene suppression analysis, a 0.4-kb fragment of *OsGWD1* was amplified using ExTaq DNA polymerase (Takara Bio Inc.), with the GWD1-L4/R2 primer pair (**Table 1**) and cDNA from green seedlings, and introduced into the RNAi transformation vector, pANDA (Miki and Shimamoto, 2004). Transgenic rice plants were generated using the *Agrobacterium tumefaciens*-mediated method described by Hiei et al. (1994), and grown in a temperature-controlled glasshouse (30/23°C, day/night).

Table 1 | List of the PCR primers used in this study.

Gene	Primer	Nucleotide sequence	Purpose*
OsGWD1	PL2	ttgccttctgttcgccttaaaa	C
	L4	ctctcccaaggtactgggt	I, S
	L17	tgaagccacgtgagataagc	S
	L18	gcttaaagggatggaatcaagc	S
	L19	gcagaagctggccaggcagt	R
	R2	gttatacatgtcccacggc	I
	R14	aacattgcctgattgacttggcta	C, S
	R23	ctgccaacttccaagctg	S
	R24	acgttgcgagacttggcccc	R
RUBIQ1	L1	ggagctgctgctgttcttgg	R
	R1	cacaatgaaacgggacacga	R

**C, gene construct for functional complementation; I, gene construct for RNAi suppression; R, real-time RT-PCR; S, analysis of the transcript structure.*

QUANTITATIVE RT-PCR ANALYSIS

For the analyses of mRNA abundance, rice plants were grown in plastic pots filled with nursery soil for rice seedlings. For tissue-specific expression analysis, rice seedlings were grown in a greenhouse under natural light conditions. For expression analyses of diurnal changes, rice plants were grown in a growth chamber with a 14-h light (27°C)/10-h dark (23°C) photoperiod. The light intensity in the light period was 350 μmol m^{-2} s^{-1} and the light was emitted by fluorescent light tubes. Once the tissue samples were obtained from the plants, they were immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was extracted from various tissue samples using the extraction buffer described by Chang et al. (1993), although polyvinylpyrrolidone and spermidine were excluded. The extracted RNA was then purified using an RNeasy Mini Kit (Qiagen) followed by a TURBO DNA-free Kit (Ambion, Austin, TX, USA) to remove DNA. The purified RNA was reverse-transcribed and then an aliquot of the first-strand cDNA mixtures corresponding to 5 ng of total RNA was used as the template for real-time quantitative reverse transcription (RT)-PCR analysis using the SuperScriptIII Platinum Two-Step qRT-PCR Kit with SYBR Green (Life Technologies, Austin, TX, USA). The reaction was carried out using an ABI7300 system (Applied Biosystems, Foster City, CA, USA) with the gene-specific primers listed in **Table 1**. In preliminary experiments, aliquots of the PCR reactions were electrophoretically separated on agarose gels to verify the specificity of the primers. The specificity of each PCR amplification was also checked using a heat dissociation protocol with temperatures changing from 60 to 95°C following the final cycle of the PCR. The results obtained for each cDNA were standardized to the expression of the rice polyubiquitin gene (*RUBIQ1*), which is a constitutively expressed gene in rice (Wang et al., 2000).

DETERMINATION OF STARCH AND SOLUBLE SUGAR CONTENTS

Frozen leaf samples were ground using a mortar and pestle under cryogenic conditions. A portion of the ground tissue (∼25 mg per sample) was immediately weighed and then extracted twice with 80% ethanol at 80°C. After centrifugation at 8500 *g* for 5 min, the supernatant was dried *in vacuo*, dissolved in distilled water, and used to assay sucrose, glucose, and fructose by the enzymatic method using F-kit #716260 (J. K. International, Tokyo, Japan), which contains an invertase, a hexokinase, a glucose 6-phosphate dehydrogenase (G6PDH), and a phosphoglucomutase. The residual pellet obtained after centrifugation was dried in air, resuspended in distilled water, gelatinized by boiling for

4 h, and used to assay starch by the enzymatic method using F-kit #207748 (J. K. International), which contains an amyloglucosidase, a hexokinase, and a G6PDH. The assays were conducted according to the manufacturer's instructions, and the increase in the absorbance at 340 nm in each assay, resulting from the enzymatic conversions of starch, sucrose, glucose, or fructose into 6-phosphogluconate, were measured with a 96-well plate reader (Viento XS; Dainippon Sumitomo Pharma, Osaka, Japan).

DETERMINATION OF THE PHOSPHORYLATION STATUS OF STARCH

Fully elongated leaf blades of 3-week-old seedlings, grown under natural light conditions, were sampled 2 h after sunset, and frozen immediately. Approximately 200 mg of frozen leaves were used in order to extract the starch, using the method as described above with slight modifications. The pellet after centrifugation was washed with distilled water three times to remove soluble metabolites completely, and resuspended in 0.5 mL distilled water. The resuspended pellet was gelatinized by boiling for 4 h; then, it was digested thoroughly to hexose units by adding 0.1 mL amyloglucosidase (TOYOBO, Tokyo, Japan) at a concentration of 50 U mL^{-1} in 50 mM sodium acetate buffer (pH 4.6) and incubating the solution at 60°C for 18 h with gentle shaking. Using the resultant digested starch samples, we first measured the amount of glucose, $[Glc]_{starch}$, by the same enzymatic method as described above. Next, as for the same digested starch samples, we also measured the amount of glucose 6-phosphate, $[G6P]_{starch}$, by another enzymatic method using highly purified G6PDH (Roche Diagnostics, Mannheim, Germany) as the sole enzyme, to allow the conversion of only G6P to 6-phosphogluconate. More than 30 μg of starch (equivalent to 185 nmol of the hexose unit) was used to assay G6P in a 200-μL reaction, in order to obtain significant increases in the absorbance at 340 nm. No significant changes in the absorbance were detected when 200 nmol of authentic glucose instead of digested starch samples were added into the 200-μL assay mixture, indicating that the hexokinase activity contamination from the G6PDH enzyme solution was negligible.

Since the enzymatic assay of $[Glc]_{starch}$ provides the total concentration of Glc plus G6P, the phosphorylation status of starch was calculated according to the following equation:

$$[G6P]_{starch}/[Glc]_{starch} \times 100 = \text{percentage of phosphorylated glucose residue in starch (\%)}.$$

MEASUREMENTS OF PHOTOSYNTHESIS RATE AND ^{13}C-PHOTOASSIMILATE PARTITIONING

Rice plants were grown in plastic pots in glasshouses until the 6th to 7th leaf stage and then used for the experiments. Net CO_2-assimilation rates in the leaf blades were measured using a portable photosynthesis system (LI-6400, LI-COR, Lincoln, NE, USA). Analysis of ^{13}C-photoassimilate partitioning in rice plants was conducted according to the method described by Sasaki et al. (2005) with modifications. In brief, $^{13}CO_2$ was fed to rice seedlings in sealed, transparent plastic box, for 2 h (from 11:00 to 13:00) under the light intensity of around 750 μmol m^{-2} s^{-1}, in a controlled glasshouse (27/23°C, 14-h day/10-h night, the day period from 5:00 to 19:00). $^{13}CO_2$ gas was liberated from $Ba^{13}CO_3$ powder (≥98 atom%, Cambridge Isotope Laboratories, Andover, MA, USA) mixed with 7.3 M H_3PO_4 inside the box. After the ^{13}C-feeding was ceased by removing the plastic box, the aboveground parts of the seedlings were sampled at designated times; immediately after the cessation of ^{13}C-feeding (13:00), in the evening of the same day (19:00), and in the morning of the next day (7:00). The aboveground samples were divided into leaf blades and sheaths, and then stored at −80°C until use. For measuring ^{13}C contents, the frozen tissue samples were freeze-dried, followed by measuring dry weight. The dried tissue materials were ground to a fine powder, and a portion (ca. 200 μg) of the tissue powder was used for the determination of ^{13}C content. The total carbon and ^{13}C contents were determined using an elemental analyzer (NC2500, Thermoquest, San Jose, CA, USA) and a mass spectrometer (Delta Plus System, Thermoquest). The ^{13}C content in each tissue sample was calculated according to the equation of Sasaki et al. (2005). Partitioning of ^{13}C-photoassimilates to the leaf blades was calculated from the total amounts of ^{13}C in leaf blades, $[^{13}C]_{LB}$, and sheaths, $[^{13}C]_{LS}$, according to the following equation:

$$[^{13}C]_{LB}/\{[^{13}C]_{LB} + [^{13}C]_{LS}\} \times 100 = {}^{13}\text{C-partitioning to leaf blades (\%)}.$$

FIELD TRIALS AND DETERMINATION OF YIELD COMPONENTS AND GRAIN YIELD

Field trials were carried out in paddy fields at the Institute for Sustainable Agro-ecosystem Services (ISAS), Tokyo, Japan (35°44′N, 139°32′E). Seeds were sown in a greenhouse in late April, and transplanted into the paddy fields in late May. In any given year, the planting density was 22.2 hills per square meter (hill spacing of 30 cm × 15 cm) with one seedling per hill, and compound fertilizer for paddy fields ($N:P_2O_5:K_2O = 12:16:18\%$) was applied at the rate of 50 g m^{-2} as a basal dressing. Approximately 45 days after the heading, plants were harvested in late September, and their panicles were used for the analysis of yield components. The panicle number per hill (A) and spikelet number per panicle (B) were counted by hands. Subsequently, ripened grains were selected as unhulled grains that settle down in a salt solution with a density of 1.06 g mL^{-1}. The percentage of ripened grains (C) was calculated from the total spikelet number per hill and the ripened grain number. Unhulled grain yield was calculated from the yield components measured and the planting density according to the following equation.

$$A \times B \times C/100 \times [\text{grain weight (g 1000-grain}^{-1})]/1000 \times 22.2(\text{hill m}^{-2}) = \text{grain yield (g m}^{-2}).$$

RESULTS

SCREENING OF THE LEAF STARCH EXCESS MUTANTS AND THE LSE1 PHENOTYPE

Of 6377 Tos17 mutant lines, 5 were selected by iodine staining to detect distinct accumulation of leaf starch (stain-positive plants). One of the five lines designated as ND0717, exhibited dark staining for starch in the entire terrestrial portion while the wild-type plants did not show any discernible staining (**Figure 1**). Because the seeds originally used for the screening were M2 seeds, i.e., those borne

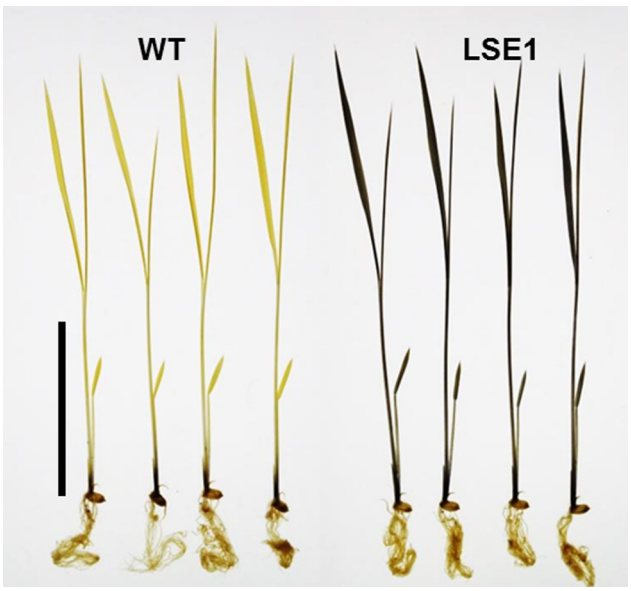

FIGURE 1 | Iodine staining of LSE1 mutant seedlings. Rice seedlings were grown for 14 days and sampled just after the onset of light period. Starch was visualized by iodine staining after the chlorophyll was removed by ethanol. Bar=5cm

on the M1 generation plants, they segregated into stain-positive and stain-negative plants. The progeny of these M2 plants were further tested for leaf starch accumulation, and in the M3 generation, the progeny of some stain-negative M2 plants segregated into stain-positive and stain-negative ones at a ratio of 1:3, suggesting that the LSE phenotype was due to the recessive mutation of a single gene. To establish homozygously mutated lines, the progeny of stain-positive M2 plants were examined to check whether all the individuals showed the stain-positive phenotype. Based on the results of this analysis, of the 15 original M2 plants, 2 were identified as having the homozygous mutant genotype, 2 as wild-type, and the remaining 11 as having the heterozygous genotype. After this, we used the progeny of the two putative homozygous mutant plants as the pure line for the mutation and designated this mutation LSE1, while the progeny of the two putative wild-type genotype plants were used as controls in the various assays in this study and were designated "wild-type." The starch content in the mature leaf blades of LSE1 plants was 5- to 10-fold higher than that in the wild-type plants throughout the day (**Figure 2A**). Contrastingly, the sucrose content in the leaf blades was not different between the two (**Figure 2B**).

GENE MAPPING OF THE LSE1 MUTATION

Southern blot analysis revealed that the LSE phenotype of LSE1 was not tagged by the Tos17 retrotransposon; it was therefore believed to be caused by some other mutation (data not shown). In order to identify the responsible gene for the mutation, gene mapping was carried out. The responsible gene was mapped to a 1.16-Mb region on chromosome 6 flanked by two genetic markers RM20061 and RM20104 (McCouch et al., 2002). Although as many as 77 genes are annotated within this region, it was significant that a gene for a putative GWD, Os06g0498400, was located in this region because disruption of the corresponding orthologs of this gene results in the LSE phenotype in both *Arabidopsis* (SEX1 mutant; Caspar et al., 1991) and *Lotus japonicus* (Vriet et al., 2010). Therefore, we decided to further explore this gene as a major candidate gene causing the LSE1 phenotype, and the gene Os06g0498400 was designated as *OsGWD1*. As the first

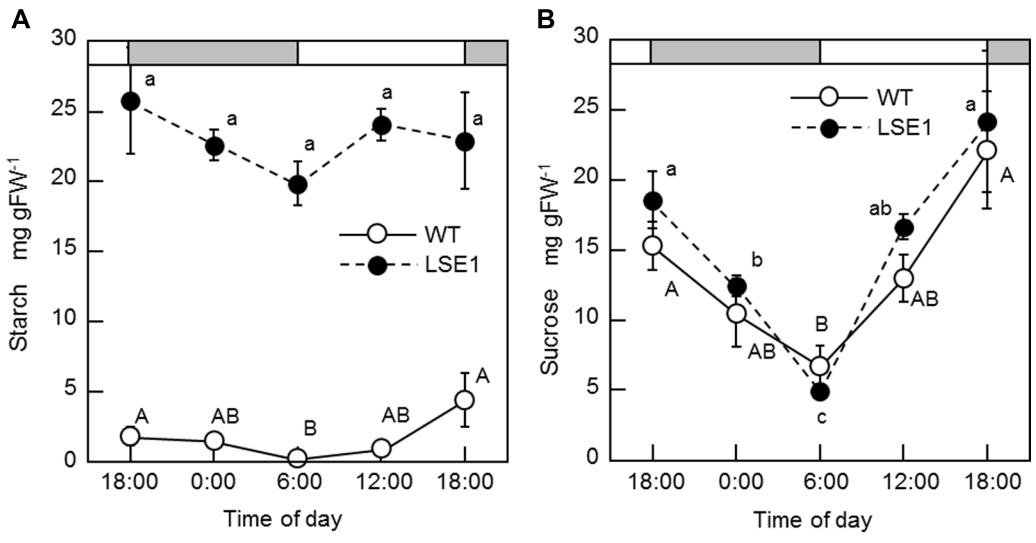

FIGURE 2 | Diurnal changes in starch (A) and sucrose (B) levels. Rice seedlings were grown in a growth chamber with a 12-h light (6:00–18:00)/12-h dark photoperiod. Temperature was maintained at 25°C during the light period and at 20°C during the dark period. White and gray bars represent light and dark periods, respectively. Fully expanded leaf blades of 3-week-old seedlings were harvested at designated times of the day and used for the measurements. Data represent the mean ± SE ($n = 5$) and the different letters indicate significant differences within each of the two lines, capital letters for WT and small letters for LSE1 ($p < 0.05$, Tukey's test).

step, we examined whether the structure of the *OsGWD1* gene was different between the LSE1 mutant and the original cultivar Nipponbare, by genomic PCR analysis using several primer combinations designed based on the published DNA sequence of Os06g0498400. *OsGWD1* has been predicted to be a relatively complex gene structure comprising as many as 32 exons, and it was found that the *OsGWD1* gene in the LSE1 mutant had a long deletion of 3434 bp corresponding to the nucleotide position from 7415 to 10758 of the reference sequence in the database, NC_008399. As a result, *OsGWD1* in the LSE1 mutant lacks the exons from the 22nd to the 32nd (**Figure 3A**).

EXPRESSION ANALYSIS OF *OsGWD1* IN LSE1 AND THE WILD-TYPE PLANTS

The transcript of *OsGWD1* in the leaf blades was analyzed in both LSE1 and the original cultivar Nipponbare by RT-PCR. According to the gene structure and the sequences of the cDNA clones in the database, the transcript of *OsGWD1* was predicted to be ∼4.4 kb in wild-type plants and to contain a 1421-bp deletion in the case of LSE1, and the primer pairs were designed to check the structure of the transcript (**Figure 3B**). With a primer pair that spanned the LSE1 deletion (L18/R14; **Figure 3C**), a shorter PCR product was amplified with the expected size in LSE1, and no PCR product was detected with a left primer that was located within the deletion (L4/R14; **Figure 3C**).

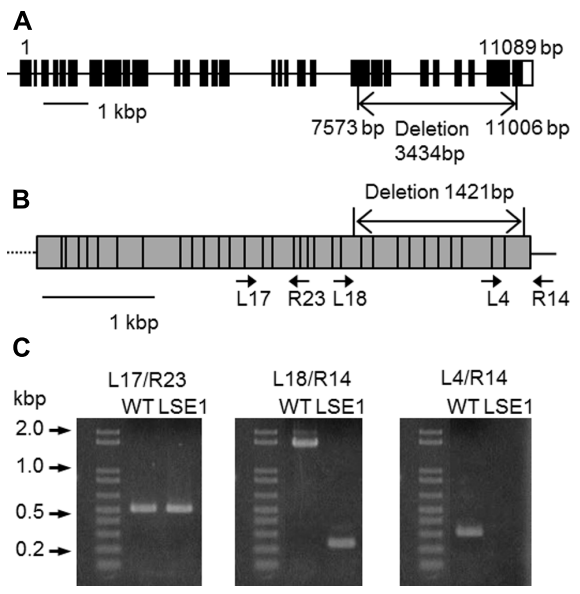

FIGURE 3 | Structure of *OsGWD1* and its transcript. (A) The gene structure of *OsGWD1* is illustrated; black boxes indicate exons and the position of the gene deletion in LSE1 mutant is shown by an arrow. **(B)** The coding region of the *OsGWD1* transcript is indicated by a gray rectangle, in which the junctions of the exons are shown by vertical bars. The positions of the PCR primers used for the analysis of the transcript structure are indicated by the arrow heads (see below). Note that the primers L17, L18, L4, and R23 span the exon/intron junction on the cDNA. **(C)** Agarose gel electrophoresis images of the PCR products are presented to demonstrate that an aberrant transcript is accumulated in the leaves of LSE1 plants. The results of PCR with the different primer pairs are shown.

To determine the expression pattern of *OsGWD1* in normal rice plants, real-time RT-PCR analysis was conducted using various tissues from 2-week-old plants and 4-day-old germinating seeds of Nipponbare. The mRNA levels in both leaf blades and sheaths were relatively high, while those in the roots were much lower (**Figure 4A**). The diurnal change in the mRNA level of *OsGWD1* was also examined using the fully expanded leaf blades of 2-week-old plants, grown with a 14-h light period (**Figure 4B**). The mRNA level of *OsGWD1* was highest in the early night (20:00) and then decreased until the next noon (12:00).

FUNCTIONAL COMPLEMENTATION OF *OsGWD1* IN THE LSE1 MUTANT

To examine whether *OsGWD1* is responsible for the LSE1 mutation, the mutant plants were introduced with a genomic DNA fragment from Nipponbare containing the entire ORF along with a 2.2-kb sequence upstream of the putative translation start point

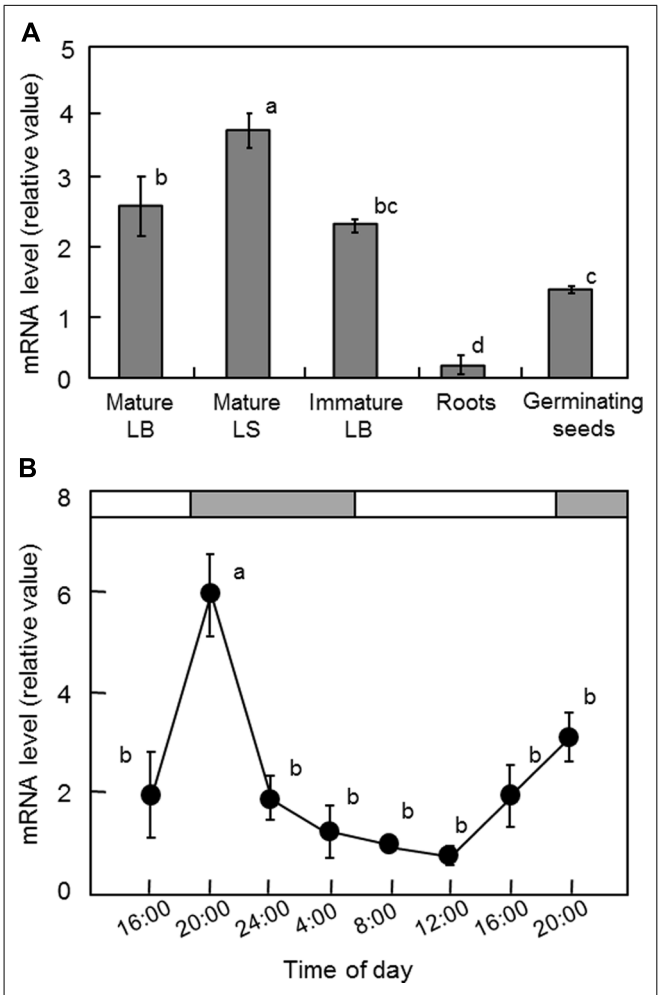

FIGURE 4 | Transcript levels of *OsGWD1* in rice seedlings. Tissue-specific accumulation pattern **(A)** and diurnal changes in leaf blades **(B)**, with respect to the mRNA levels of *OsGWD1* were examined by quantitative RT-PCR analysis. Values are standardized to the expression level of a rice polyubiquitin gene (% *RUBIQ1*). Data represent the mean ± SE ($n = 4$) and the different letters indicate significant differences ($p < 0.05$, Tukey's test). LB, leaf blade; LS, leaf sheath.

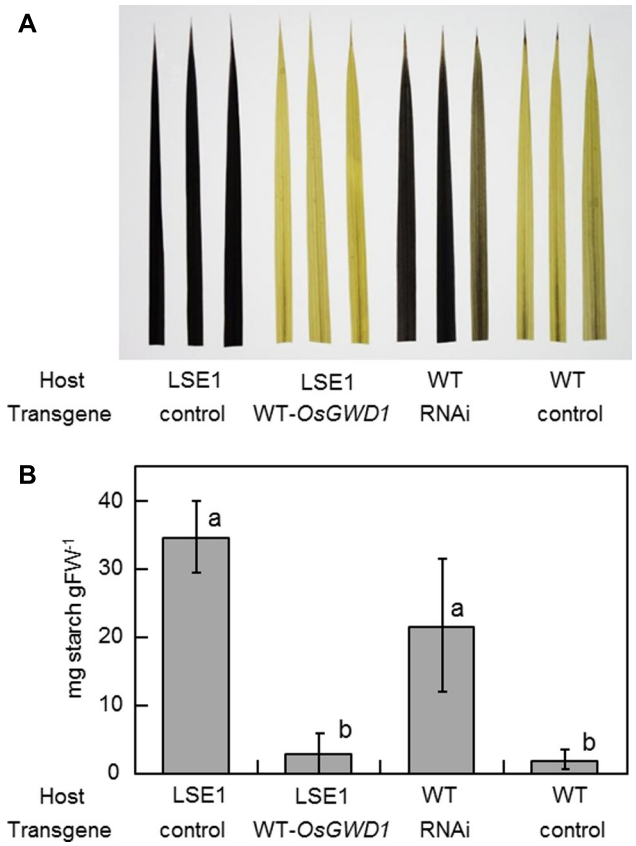

FIGURE 5 | Functional complementation of LSE1 mutation and RNAi suppression of *OsGWD1* in wild-type plants. The uppermost fully elongated leaf blades were sampled from the transgenic rice plants at the panicle formation stage. The combinations of the host and the transgene are specified below the panel. Empty vectors were introduced as the controls for each assay. Accumulation of starch was **(A)** visualized by iodine in three independent transgenic plants or **(B)** determined enzymatically using the samples from six independent transgenic plants for each combination. Data represent the mean ± SE ($n = 6$) and different letters indicate significant differences ($p < 0.05$, Tukey's test).

of *OsGWD1*. As expected, it was observed that *OsGWD1* from Nipponbare restored the LSE1 phenotype to wild-type; the starch content in the leaf blades of the transgenic LSE1 plants was lower and was similar to that seen in the controls (**Figure 5**). In addition, when the RNAi construct for *OsGWD1* was introduced into Nipponbare plants, the starch level in the leaf blades increased significantly compared to that in the controls, mimicking the phenotype of LSE1 (**Figure 5**).

PHOSPHORYLATION STATUS OF STARCH IN LSE1 MUTANT

Since GWD has been reported to play a role in the degradation pathway of starch, by catalyzing the phosphorylation of the C6 position of the glucosyl residue in amylose chains (see Blennow et al., 2002 for a review), we examined the extent of phosphorylation of the glucosyl residues in starch, by measuring the amount of G6P as well as that of glucose in digested starch extracted from mature leaf blades. Leaf samples were harvested 2 h after the sunset, during which the mobilization of leaf starch would occur actively. In wild-type leaves, the percentage of phosphorylated glucosyl residues in the starch molecules was 0.20 ± 0.04% (average ± standard error, $n = 4$), namely, 2 of every 1000 glucosyl residues were phosphorylated, which is comparable with the reported phosphorylation status of starch in *Arabidopsis* and potato plants (Yu et al., 2001; also see Blennow et al., 2002 for a review). In LSE1 leaves, the percentage of phosphorylation was 0.05 ± 0.02%, significantly lower than the wild-type leaves ($p < 0.001$ by t-test, $n = 4$).

VEGETATIVE GROWTH, LEAF PHOTOSYNTHESIS, AND ^{13}C-PHOTOASSIMILATE PARTITIONING

The vegetative growth of LSE1 was assessed by the leaf number on the main stem and the shoot dry weight. For both growth parameters, there was no significant difference between LSE1 and the wild-type control (**Figure 6A**). The rates of net photosynthesis in the leaf blades, measured under either ambient or saturating concentrations of CO_2, were not significantly different between LSE1 and the wild-type (**Figure 6B**). The effect of the LSE1 mutation on the photoassimilate partitioning was accessed by ^{13}C-tracer technique. Just after the cessation of the feeding (0-h), approximately 70% of the ^{13}C remained in the leaf blades in both genotypes, and then it decreased gradually. The ^{13}C-partitioning to the leaf blades at 6- and 18-h after feeding showed a tendency to be greater in LSE1 than in the wild-type, but the differences were not significant (**Figure 6C**).

YIELD TRAITS OF LSE1

To investigate the impact of the LSE1 mutation on grain productivity in rice plants, LSE1, wild-type, and the original cultivar Nipponbare were grown in paddy fields in Tokyo and Niigata, Japan, from 2009 to 2012. Essentially the same results were obtained in all the field trials, and **Table 2** shows a representative result obtained in 2010 in Tokyo. Among the yield components of rice, the panicle number per hill, percentage of ripened grain, and 1000-grain weight were significantly lower in LSE1 plants than in wild-type and Nipponbare plants. As a result, grain yield reductions of 20∼40% were observed in LSE1 compared with the control lines, in all the field trials conducted.

DISCUSSION

To date, a number of mutants have been reported for the hyper-accumulation of starch in their leaves. In maize, several mutant lines exhibiting the LSE phenotype have already been reported, for example, Sxd1 (Russin et al., 1996), Tdy1 (Braun et al., 2006), and Psc (Slewinski and Braun, 2010), all of which are related to photoassimilate export from the leaves. In rice, however, no such mutation has been documented so far; to our knowledge, LSE1 is the first such case. Gene mapping and gene structure analysis revealed that the LSE1 mutant has a deletion of as much as 3.4 kb in *OsGWD1*, a putative gene for α-glucan water dikinase, and consequently, accumulates aberrant mRNA lacking one-third of the coding region in the leaf blades (**Figure 3**). Therefore, LSE1 was assumed to be a loss-of-function mutant of *OsGWD1*. This hypothesis was strongly supported by the complementation test for the LSE1 mutation with an intact *OsGWD1* and by RNAi-mediated suppression of *OsGWD1* in Nipponbare plants (**Figure 5**). Disruption of the genes for GWD has already

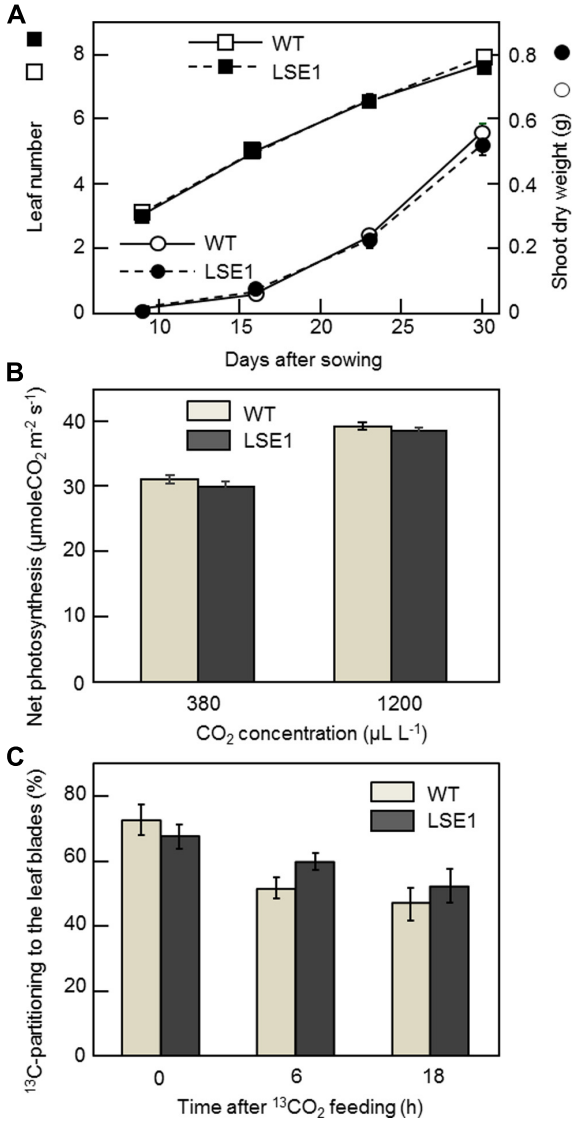

FIGURE 6 | Growth, photosynthesis and ^{13}C-photoassimilate partitioning in the seedlings of LSE1. (A) The dry weight of the terrestrial portion (circles) and the leaf number (squares) were examined in the seedlings of LSE1 (closed symbols) and wild-type plants (open symbols) during the early vegetative growth stage. Data represent the mean ± SE ($n = 5$). **(B)** Net photosynthesis rates of the uppermost fully elongated 7th leaf blades were measured under CO_2 concentrations of both 380 and 1200 μL L^{-1}. Light intensity was set at 1200 μmol photon m^{-2} s^{-1}. Data represent the mean ± SE ($n = 4$). **(C)** Partitioning of ^{13}C-photoassimilate to the leaf blades was measured immediately (0 h), 6 h, and 18 h after the cessation of 2-h feeding of $^{13}CO_2$. The seedlings at the 6th leaf stage were used in the experiment. Data represent the mean ± SE ($n = 4$).

been reported to lead to the LSE phenotype in *Arabidopsis* (SEX1; Caspar et al., 1991), *L. japonicus* (Vriet et al., 2010), and tomato (Nashilevitz et al., 2009), all of which are observed to show LSE phenotype.

In normal plants, a higher level of the *OsGWD1* transcript was observed in leaf blades and sheaths but a much lower level was seen in roots (**Figure 4A**), showing agreement with the pattern of starch accumulation in LSE plants visualized by iodine staining (**Figure 1**). The transcript level of *OsGWD1* in leaf blades changed diurnally and peaked just after the onset of the dark period (**Figure 4B**), which seems reasonable as it is believed to play a role in the degradation of starch accumulated during the light period. A similar diurnal change in the GWD transcript level has been reported in *Arabidopsis* (Yu et al., 2001; Smith et al., 2004). However, GWD protein level was shown not to change diurnally (Yu et al., 2001; Lu et al., 2005). Further investigation including diurnal change in GWD activity is needed to better understand this problem. The biochemical and physiological functions of GWD have been extensively studied in the *Arabidopsis* SEX1 mutant and are now believed to involve phosphorylation of the glucosyl residues in starch, which facilitates amylolytic degradation (see Blennow et al., 2002 for a review). In the present study, it was observed that leaf starch from the LSE1 plants contained less G6P than that from wild-type plants, consistent with observations in *Arabidopsis* SEX1 (Yu et al., 2001). Accordingly, *OsGWD1* appears to fulfill the same role in the process of leaf starch degradation in rice as in other plant species in which disruption mutants of GWD genes are reported. In other words, it was suggested that GWD has universal importance in the degradation of leaf starch among higher plants.

While LSE1 shares considerable similarity in its phenotype with the reported mutants of GWD, a conspicuous difference was found in the vegetative growth; LSE1 grows normally unlike the GWD mutants of *Arabidopsis* and *L. japonicus* (**Figure 6A**). The reason for this difference is not clear. However, it may be relevant to the physiological priority of leaf starch in the photoassimilates. In rice leaf blades, newly fixed carbon is preferentially directed to sucrose biosynthesis rather than starch, in contrast to other plant species such as *Arabidopsis*. This is evident from the fact that the content of starch and sucrose in rice leaf blades at the end of the day is ca. 5 and 20 mg per gram flesh weight (gFW^{-1}), respectively, while that reported for *Arabidopsis* in the literatures is around 10 and 1 mg gFW^{-1}, respectively (**Figures 2A,B**; e.g., Chia et al., 2004). Accordingly, rice may have sufficient capacity for the synthesis of sucrose to achieve normal growth without the transient starch in the chloroplast, and thus may experience limited impact on growth when the mobility of the transient starch is impaired by a lesion in *OsGWD1*. This view is reinforced by the observation that a knockout mutant of the ADP-glucose pyrophosphorylase (AGPase) gene, *OsAPL1*, grows normally although it accumulates less than 5% of starch in the leaf blades in comparison with wild-type plants (Rösti et al., 2007). In addition, the sucrose synthesized in photosynthetic cells was considered to be exported from the source leaves of LSE1 as efficiently as wild-type, from the facts that (1) bulk concentration of sucrose in the leaf blades did not differ between the two genotypes throughout the day (**Figure 2B**), and (2) photoassimilate export estimated by decrease in the ^{13}C-partitioning to the leaf blades after the tracer feeding was also comparable between the two (**Figure 6C**). Finally, the present results indicate that hyperaccumulation of starch in the source leaves does not inhibit photosynthesis in the leaves (**Figure 6B**), which has often been assumed in the research history of the regulation of photosynthesis (see Stitt, 1991 for a review).

Table 2 | Yield components and grain yield of LSE1, wild-type and the original cultivar Nipponbare grown under field conditions.

	Panicle number per plant	Spikelet number per panicle	Percentage of ripened grain	Grain weight (g 1000 grains^{-1})	Grain yield (g m^{-2})
Wild-type	9.5 ± 0.2 (100)	105.8 ± 3.0 (100)	74.8 ± 1.6 (100)	26.5 ± 0.3 (100)	444.1 ± 8.9 (100)
LSE1	8.7 ± 0.3* (91)	101.1 ± 4.0 (96)	64.1 ± 2.0** (86)	25.1 ± 0.2** (95)	313.2 ± 21.2** (71)
Nipponbare	9.0 ± 0.3 (95)	115.8 ± 4.8 (109)	78.9 ± 0.8 (105)	26.7 ± 0.1 (101)	488.9 ± 31.0 (110)

*Data were obtained in the field trial, conducted at The University of Tokyo in 2010. The numbers in parentheses indicate relative values when the mean value in the wild-type is 100. Data represent the mean values ± SE (n = 12). Asterisks indicate the significance of the mean values of LSE1 plants or Nipponbare plants from that of wild-type plants by t-test at *p < 0.05 and **p < 0.01. Note that no significant differences were observed in yield components and grain yield between wild-type plants and the original cultivar Nipponbare, indicating that these two lines were similar with regard to growth and productivity in paddy fields.*

Recently Weise et al. (2012) reported in maize that RNAi suppression of a GWD gene did not affect the vegetative growth in spite of a high level of starch accumulation in leaves. This, together with our results, suggests that vegetative growth of grass species is less dependent on GWD-mediated leaf starch remobilization than the dicot species reported.

Nevertheless, it should be also noted that the grain yield of LSE1 was significantly lower than that of wild-type plants and Nipponbare because of combined suppression of yield components (**Table 2**). This finding indicates that the impairment of GWD exerted some adverse effect on reproductive growth, although such effects are not apparent in vegetative growth. It is possible that the impairment of GWD in LSE1 slightly affected growth throughout the lifecycle in a manner that was not detectable in the short-term, and the significant reduction in the yield traits might be a manifestation of the cumulative effects. Information on the effect of GWD mutation on the reproductive growth is still limited, although Andriotis et al. (2012) recently reported that mature seeds of *Arabidopsis* SEX1 mutant have less lipid content than seeds of wild-type plants due to starvation of the carbohydrate in the fruit imported from the maternal plant. Here again, mutant plants lacking leaf starch can be a good reference because they share unavailability of leaf starch in common with LSE1. However, the results reported are inconsistent; AGPase mutants lacking leaf starch, rice *apl1* (Rösti et al., 2007) and maize *agps-m1* (Slewinski et al., 2008) showed no reduction in productivity under controlled environment whereas the latter mutant did exhibit yield reduction when grown in the field (Schlosser et al., 2012). It may be possible that the adverse effects of the mutation become apparent only in the field where plants routinely experience various environmental stresses unlike in the greenhouse. It may be also assumed that in LSE1 some disorder in starch metabolism in the stem reserve was brought about by the mutation because the stems of rice accumulate starch before heading to meet a large demand of carbohydrate for grain filling and also to buffer unfavorable environmental conditions after heading (see Slewinski, 2012 for a review). In our preliminary experiment, however, the stem starch content at the heading stage did not differ between LSE1 and the wild-type plants (data not shown), although the effects of the mutation on the mobilization of stem starch after heading are yet to be elucidated. A more extensive study with an agronomical perspective is clearly needed to uncover the mechanism of yield reduction in LSE1.

In conclusion, the LSE1 mutation in rice, i.e., disruption of *OsGWD1*, resulted in a mild phenotypic change at the whole plant level, highlighting the variable physiological priority of leaf starch among plant species. In addition to LSE1, we have isolated additional Tos17 mutant lines exhibiting the LSE phenotype, and some of these LSE mutants appear to exhibit a severe phenotype with respect to growth (Hirose, Aoki, Miyao, Hirochika, unpublished). Analysis of the LSE mutants as well as the identification of the responsible genes would increase our understandings of the molecular mechanism and/or physiological significance of photoassimilate allocation in rice.

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REFERENCES

Andriotis, V. M. E., Pike, M. J., Schwarz, S. L., Rawsthorne, S., Wang, T. L., and Smith, A. M. (2012). Altered starch turnover in the maternal plant has major effects on *Arabidopsis* fruit growth and seed composition. *Plant Physiol.* 160, 1175–1186.

Blennow, A., Engelsen, S., Nielsen, T., Baunsgaard, L., and Mikkelsen, R. (2002). Starch phosphorylation: a new front line in starch research. *Trends Plant Sci.* 7, 445–450.

Braun, D. M., Ma, Y., Inada, N., Muszynski, M. G., and Baker, R. F. (2006). *tie-dyed1* regulates carbohydrate accumulation in maize leaves. *Plant Physiol.* 142, 1511–1522.

Bürkle, L., Hibberd, J. M., Quick, W. P., Kühn, C., Hirner, B., and Frommer, W. B. (1998). The H+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. *Plant Physiol.* 118, 59–68.

Caspar, T., Lin, T. P., Kakefuda, G., Benbow, L., Preiss, J., and Somerville, C. (1991). Mutants of *Arabidopsis* with altered regulation of starch degradation. *Plant Physiol.* 95, 1181–1188.

Chang, S., Puryear, J., and Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11, 113–116.

Chia, T., Thorneycroft, D., Chapple, A., Messerli, G., Chen, J., Zeeman, S. C., et al. (2004). A cytosolic glucosyltransferase is required for conversion of starch to sucrose in *Arabidopsis* leaves at night. *Plant J.* 37, 853–863.

Critchley, J. H., Zeeman, S. C., Takaha, T., Smith, A. M., and Smith, S. M. (2001). A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in *Arabidopsis*. *Plant J.* 26, 89–100.

Gottwald, J. R., Krysan, P. J., Young, J. C., Evert, R. F., and Sussman, M. R. (2000). Genetic evidence for the in planta role of phloem-specific plasma membrane sucrose transporters. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13979–13984.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L) mediated by *Agrobacterium* and sequence-analysis of the boundaries of the T-DNA. *Plant J.* 6, 271–282.

Krapp, A., Hofmann, B., Schafer, C., and Stitt, M. (1993). Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates – a mechanism for the sink regulation of photosynthesis. *Plant J.* 3, 817–828.

Kuroda, M., Kimizu, M., and Mikami, C. (2010). A simple set of plasmids for the production of transgenic plants. *Biosci. Biotechnol. Biochem.* 74, 2348–2351.

Lu, Y., Gehan, J. P., and Sharkey, T. D. (2005). Daylength and circadian effects on starch degradation and maltose metabolism. *Plant Physiol.* 138, 2280–2291.

McCouch, S. R., Teytelman, L., Xu, Y. B., Lobos, K. B., Clare, K., Walton, M., et al. (2002). Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res.* 9, 199–207.

Miki, D., and Shimamoto, K. (2004). Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol.* 45, 490–495.

Miyao, A., Tanaka, K., Murata, K., Sawaki, H., Takeda, S., Abe, K., et al. (2003). Target site specificity of the Tos17 retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome. *Plant Cell* 15, 1771–1780.

Nashilevitz, S., Melamed-Bessudo, C., Aharoni, A., Kossmann, J., Wolf, S., and Levy, A. (2009). The *legwd* mutant uncovers the role of starch phosphorylation in pollen development and germination in tomato. *Plant J.* 57, 1–13.

Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A. M., and Zeeman, S. C. (2004). A previously unknown maltose transporter essential for starch degradation in leaves. *Science* 303, 87–89.

Rösti, S., Fahy, B., and Denyer, K. (2007). A mutant of rice lacking the leaf large subunit of ADP-glucose pyrophosphorylase has drastically reduced leaf starch content but grows normally. *Funct. Plant Biol.* 34, 480–489.

Russin, W. A., Evert, R. F., Vanderveer, P. J., Sharkey, T. D., and Briggs, S. P. (1996). Modification of a specific class of plasmodesmata and loss of sucrose export ability in the *sucrose export defective 1* maize mutant. *Plant Cell* 8, 645–658.

Sasaki, H., Aoki, N., Sakai, H., Hara, T., Uehara, N., Ishimaru, K., et al. (2005). Effect of CO_2 enrichment on the translocation and partitioning of carbon at the early grain-filling stage in rice (*Oryza sativa* L.). *Plant Prod. Sci.* 8, 8–15.

Schlosser, A., Martin, J., Hannah, L., and Giroux, M. (2012). The maize leaf starch mutation *agps-m1* has diminished field growth and productivity. *Crop Sci.* 52, 700–706.

Slewinski, T. (2012). Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. *J. Exp. Bot.* 63, 4647–4670.

Slewinski, T., and Braun, D. (2010). The *psychedelic* genes of maize redundantly promote carbohydrate export from leaves. *Genetics* 185, 221–232.

Slewinski, T., Ma, Y., Baker, F., Huang, S., Meeley, R., and Braun, D. (2008). Determining the role of *Tie-dyed1* in starch metabolism: epistasis analysis with a maize ADP-glucose pyrophosphorylase mutant lacking leaf starch. *J. Hered.* 99, 661–666.

Smith, S. M., Fulton, D. C., Chia, T., Thorneycroft, D., Chapple, A., Dunstan, H., et al. (2004). Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis* leaves. *Plant Physiol.* 136, 2687–2699.

Stitt, M. (1991). Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. *Plant Cell Environ.* 14, 741–762.

Vriet, C., Welham, T., Brachmann, A., Pike, M., Pike, J., Perry, J., et al. (2010). A suite of *Lotus japonicus* starch mutants reveals both conserved and novel features of starch metabolism. *Plant Physiol.* 154, 643–655.

Wang, H., Qi, M., and Cutler, A. (1993). A simple method of preparing plant-samples for PCR. *Nucleic Acids Res.* 21, 4153–4154.

Wang, J. L., Jiang, J. D., and Oard, J. H. (2000). Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). *Plant Sci.* 156, 201–211.

Weise, S. E., Aung, K., Jarou, Z. J., Mehrshahi, P., Li, Z. R., Hardy, A. C., et al. (2012). Engineering starch accumulation by manipulation of phosphate metabolism of starch. *Plant Biotechnol. J.* 10, 545–554.

Yu, T. S., Kofler, H., Hausler, R. E., Hille, D., Flügge, U. I., Zeeman, S. C., et al. (2001). The *Arabidopsis* sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell* 13, 1907–1918.

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