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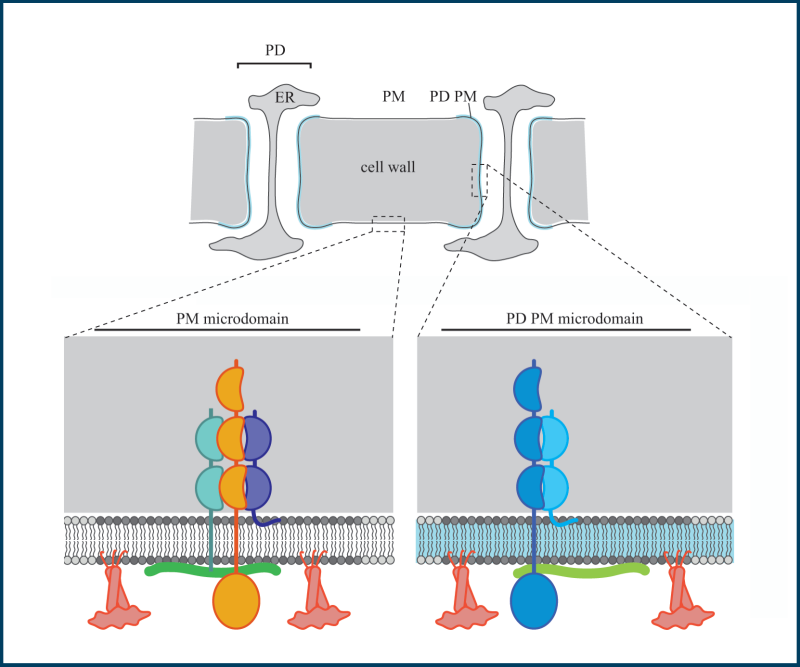

SPECIALISED MEMBRANE DOMAINS OF PLASMODESMATA, PLANT INTERCELLULAR NANOPORES

Topic Editors
Jens Tilsner, Emmanuelle Bayer,
Sébastien Mongrand and Lesley Torrance

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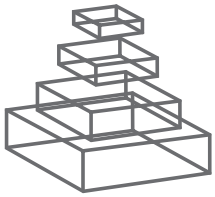

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SPECIALISED MEMBRANE DOMAINS OF PLASMODESMATA, PLANT INTERCELLULAR NANOPORES

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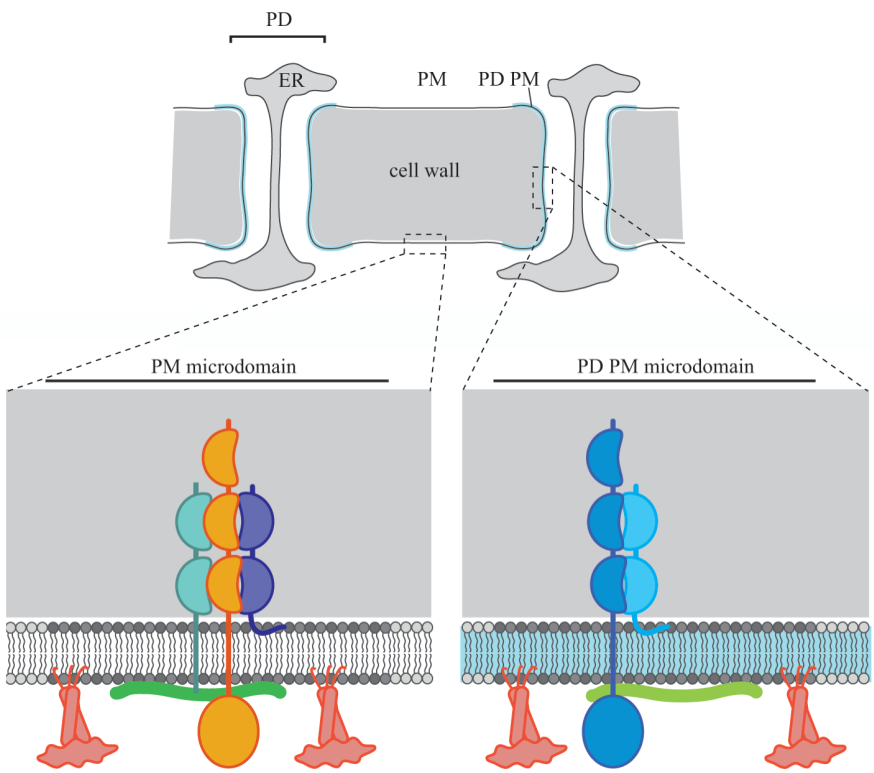

A model for the spatial separation of LysM receptor signaling at the plasma membrane and in plasmodesmata. Figure modified from Faulkner (2013) Receptor-mediated signaling at plasmodesmata. *Front. Plant Sci.* 4, 521. doi: 10.3389/fpls.2013.00521. Courtesy of C. Faulkner.

Plasmodesmata (PD) are plant-specific intercellular nanopores defined by specialised domains of the plasma membrane (PM) and the endoplasmic reticulum (ER), both of which contain unique proteins, and probably different lipid compositions than the surrounding bulk membranes. The PD membranes form concentric tubules with a minimal outer diameter of only 50 nm, and the central ER strand constricted to ~10-15 nm, representing one of the narrowest stable membrane tubules in nature. This unique membrane architecture poses many biophysical, structural and functional questions.

PM continuity across PD raises the question as to how a locally confined membrane site is established and maintained at PD. There is increasing evidence that the PM within PD may be enriched in membrane 'rafts' or TET web domains. Lipid rafts often function as signalling platforms, in line with the emerging view of PD as central players in plant defense responses. Lipid-lipid immiscibility could also provide a mechanism for membrane sub-compartmentalisation at PD. Intricate connections of the PM to the wall and the underlying cytoskeleton and ER may anchor the specialised domains locally.

The ER within PD is even more strongly modified. Its extreme curvature suggests that it is stabilised by densely packed proteins, potentially members of the reticulon family that tubulate the cortical ER. The diameter of the constricted ER within PD is similar to membrane stalks in dynamin-mediated membrane fission during endocytosis and may need to be stabilised against spontaneous rupture. The function of this extreme membrane constriction, and the reasons why the ER is connected between plant cells remain unknown.

Whilst the technically challenging search for the protein components of PD is ongoing, there has been significant recent progress in research on biological membranes that could benefit our understanding of PD function. With this Research Topic, we therefore aim to bring together researchers in the PD field and those in related areas, such as membrane biophysics, membrane composition and fluidity, protein-lipid interactions, lateral membrane heterogeneity, lipid rafts, membrane curvature, and membrane fusion/fission.

We wish to address questions such as:

- What mechanisms restrict lateral mobility of proteins and lipids along the PD membranes?
- How can specific proteins be targeted to and turned over from membrane domains with restricted lateral access?
- What elements (lipids, proteins, membrane curvature, packing order, thickness etc.) may contribute to the identity of PD membranes?
- How do the structural and functional features of PD compare to other ER-PM contact sites?
- How is the high curvature of the PD ER stabilised and what are possible functions of such a tightly constricted membrane tubule?
- Do PD need to be prevented from spontaneous collapse and sealing?
- What technologies are available to address these questions that can underpin PD research?

We welcome interested individuals to contribute their expertise and develop new hypotheses on the particular biological and biophysical questions posed by PD. We are particularly looking for articles (Original Research Articles, Technical Advances and State-of-the-Art reviews) that would expand on or challenge current perceptions of PD and stimulate discussion.

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EDITORIAL
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Specialized membrane domains of plasmodesmata, plant intercellular nanopores

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Keywords: plasmodesmata, lipid rafts, membrane microdomains, membrane curvature, plasma membrane, endoplasmic reticulum, super-resolution microscopy, protein-lipid interaction

Plasmodesmata (PD) are plant-specific membrane-lined channels that connect neighboring cells across the cell wall and are indispensable for intercellular communication, development and defense against pathogens. They consist of concentric membrane tubules of the plasma membrane (PM) on the outside and endoplasmic reticulum (ER) on the inside. The biophysical properties and molecular composition of both membranes are most likely distinct from the respective bulk membranes with which they are continuous. This specialization of PD membranes is expected to guarantee not only the compartmentalization of PD-related function but also to accommodate the requirement for highly curved membrane organization (Mongrand et al., 2010; Tilsner et al., 2011).

This Research Topic brings together researchers from a variety of areas to apply the significant recent advances in understanding how the interactions of lipids and proteins influence the behavior and spatial/functional compartmentalization of biological membranes on PD-related questions.

The first several contributions are focussed on the molecular and physical properties of the PD plasma membrane (PD-PM). The PD-PM contains a different set of proteins than the PM outside the channels and does not permit free diffusion of membrane components between cells, indicating that it is laterally segregated from the bulk PM and forms a membrane microdomain (or several). In line with a view of microdomains as signaling "hubs," PD have recently been emerging as important sites of pathogen-related and developmental signaling. Faulkner (2013) reviews the currently identified PD-located receptors and suggests that sub-division of the PD-PM into microdomains, be it raft-like or tetraspanin webs, may facilitate signaling processes through the local clustering of membrane components. Preferential compartmentation of proteins but also lipids into membrane microdomains have been postulated for many cell types, but have long been difficult to directly visualize *in vivo*. Owen and Gaus (2013) and Truong-Quang and Lenne (2014) both review how recent advantages in light microscopy that allow imaging below the diffraction limit can be used to obtain new insights into the dynamics of microdomains and to draw conclusions on the mechanism of their formation. Truong-Quang and Lenne review internal structuring as well as higher-order clustering of microdomains. Owen and Gaus discuss their recent findings from direct imaging of PM lipid order *in vivo*. They found the PM to consist of ~75% liquid-ordered (L_o) and 25% liquid-disordered (L_d) sub-resolution microdomains and postulate that small changes in lipid phase distribution can induce rapid large-scale changes in protein geometry of the PM when a lipid phase switches from being the "island" to the "percolating" phase and vice versa.

So far no data exist as to how lateral membrane heterogeneity and compartmentalization of biological processes are achieved at PD. In other words, how are locally confined PD membrane sites established and maintained within the pore, despite their continuity with the bulk membranes outside PD? What mechanisms restrict lateral mobility of proteins and possibly lipids along the PD membranes?

A number of articles ask how a laterally segregated PM domain could be maintained at PD (and elsewhere). One potential mechanism for microdomain formation is the "picket fence" model which suggests that, in mammalian cells at least, PM domains are corralled by structural elements attached to the membrane and underlying cytoskeleton. In plant cells different mechanisms might be at work. Martinière and Runions (2013) review their recent experimental findings showing that compared to animal cells, most of the plant PM-resident proteins display a low mobility and that restricted lateral diffusion depends mostly on the cell wall. Intricate connections between the PD-PM and surrounding wall have been observed and are likely to contribute to the specialization of this membrane domain.

Boutté and Moreau (2014) review the role of small GTPases in PM partitioning and suggest that such mechanisms could also act at PD. Several small GTPases have been found in the PD proteome and could potentially be involved in specifying the PD-PM.

In line with the idea that the PD-PM may cluster L_o sterol and sphingolipid enriched raft microdomains, a number of articles provide insights about the potential contribution of lipid phase separation to the selective lateral segregation of PD components. de Almeida and Joly (2014) suggest that nano-scale lipid phase separation may also include the formation of solid-ordered/gel (S_o) phases around nucleating oligomers of membrane-integral proteins or lipids, which could stabilize membrane microdomains for longer time spans than the L_o domains of the conventional raft hypothesis. Whilst still speculative at this stage, such a model could potentially provide an explanation for the restricted lateral diffusion within the PD-PM. On their side Bagatolli and

Mouritsen (2013) ask whether the now-classical fluid mosaic model, together with the raft hypothesis based on segregation of L_o and L_d phases is really suitable to describe the lateral segregation that has been observed in biological and model membranes. They conclude that the molecular shape of membrane lipids, which introduces curvature stress into bilayers, needs to be taken into consideration when investigating membrane lateral segregation, and the distribution and activity of proteins in the bilayer.

Although the involvement of lipids in PD functionality has been suggested, we actually know very little about PD lipid constituents. As a way to determine whether sterol- and sphingolipid-enriched microdomains contribute to PD membranes as suggested by the presence of raft and tetraspanin protein markers, Naulin et al. (2014) propose an original approach based on Mass Spectrometry (MS) and Atomic Force Microscopy (AFM) directly on purified PD membranes. Whereas AFM could be applied to identify microdomains based on topological parameters, MS approaches could resolve the PD lipid profile and identify intact membrane proteins and the stoichiometry and nature of lipids bound to them.

In animal cells, the recently discovered tunneling nanotubes (TNTs) also connect the PM between cells via a highly curved membrane tubule, although they differ from PD by the lack of an ER connection. Delage and Zurzolo (2013) review the current knowledge of the lipid composition of TNTs and suggest that comparisons with PD lipids will be informative in understanding the functional similarities and differences between these structures. They suggest that lipids may play a critical role in the formation and stability of these highly curved structures.

Because sphingolipids are typically enriched in raft-like microdomains, González-Solís et al. (2014) describe Arabidopsis mutants defective in specific steps of sphingolipid synthesis which could be used to dissect the contribution of sphingolipids to putative PD-PM microdomains. Sphingoid bases, breakdown products of sphingolipids, also act as second messengers in pathogen-induced programmed cell death and the authors suggest that like other defense-related signaling this pathway could act at PD.

Linking lipids and the regulation of PD permeability, De Storme and Geelen (2014) discuss the potential involvement of sterols in the regulation of PD aperture through the deposition and removal of the water-insoluble cell wall polysaccharide callose (β-1,3-glucan) at the neck region of the pore, which leads to a physical constriction of the PD opening. Callose synthases are transmembrane complexes like cellulose synthases, and callose-degrading β-1,3-glucanases are membrane-anchored apoplastic enzymes. The site-specific turnover of callose at PD therefore also requires targeting of proteins and membranes to the specific domain at PD. De Storme and Geelen provide an overview of callose metabolism at PD and note similarities in the developmental phenotypes of mutations in callose synthases with those of sterol synthesis mutants. They speculate that structural sterols may play a role in PD callose turnover, either as constituents of the PD-PM, or as substrates for callose synthase.

Through phylogenetic analyses, Gaudioso-Pedraza and Benitez-Alfonso (2014) identify a subgroup of the Glycosyl hydrolases family 17 (GHL17), called clade alpha, that have diverged during plant land colonization and would therefore correlate with the appearance of complex PD. Hence, all the callose degrading enzymes identified so far belong to this specific clade. They suggest that a portion of the alpha clade GHL17 membrane proteins have evolved in embryophytes differently from other clades to specifically target PD.

Taking advantage of the growing understanding of callose turnover at PD, Yadav et al. (2014) present their *icals3m* system, which enables inducible overaccumulation of callose at PD and a concomitant reduction in intercellular communication, to study the role of PD during development. Given the intricate links between membrane trafficking to the PD-PM and callose metabolism at the pores, this system could perhaps also be combined with lipid biosynthesis mutants to investigate the targeting mechanism of PD-specific callose synthesis.

One possible way for delivery of β-1,3-glucanases to PD is suggested by Paul et al. (2014). These authors have found that lipid bodies, ER-derived structures surrounded by a lipid monolayer, are positioned at PD openings in meristematic cells prior to the removal of callose plugs at the onset of dormancy release. These lipid bodies carry β-1,3-glucanases which are probably delivered to PD bypassing the conventional secretory pathway, possibly by interacting with cytofacial plasma membrane rafts. Paul et al. speculate that a similar mechanism might also function in the delivery of other PD components.

PD are dynamically modified during plant development, and Demchenko et al. (2014) present their research on specialized cells hosting actinorhizal, nitrogen-fixing bacteria in *Casuarina* nodules, where PD become very narrow and embedded in a lignified cell wall. It appears that this modification is connected to a loss of some of the specialized membrane features of PD as the desmotubule may be removed and the wall surrounding the PD-PM is devoid of callose. Whether these modifications represent stages of PD degradation and permanent closure, or to the contrary the transformation of PD into unregulated open channels, remains to be seen.

Shifting the focus from the PD-PM to the PD-ER, the desmotubule needs to be connected to the cortical ER at either side. If this connection severs, or when new secondary PD are laid down in existing walls, the PD-ER needs to be (re)connected to the cortical ER outside of the channel. A family of proteins called atlastins was recently identified which mediates this type of homotypic ER tubule fusion. Zhang and Hu (2013) review the current knowledge about plant atlastins, also known as RHD3s.

The ability to connect the ER across the cell wall may be a precondition for the ability to form secondary PD across existing cell walls. Evkaikina et al. (2014) review the evolutionary origins of secondary PD formation and its link to the organization of the shoot apical meristem, which uses transport of miRNAs and transcription factors through PD to establish polarity axes. It is likely that the ability to form new PD involved evolution of both the cell's membrane organizing and cell wall modifying machineries.

The desmotubular ER is extremely constricted, to about the dimensions of a vesicle fission stalk, and in EM images sometimes seems to contain an electron-dense "central rod" that may correspond to lipid headgroups in a virtually lumen-less tubule.

Certain types of lipids could favor such an arrangement. Jouhet (2013) reviews the curvature-related biophysical properties of membrane lipids and highlights the possibility that the desmotubular ER may assume an unusual, non-bilayer lipid phase.

We hope that the articles collected in this Research Topic/e-book will stimulate discussion and new experimental approaches in plasmodesmata research.

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Receptor-mediated signaling at plasmodesmata

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Plasmodesmata (PD) generate continuity between plant cells via the cytoplasm, endoplasmic reticulum (ER) and plasma membrane (PM), allowing movement of different classes of molecules between cells. Proteomic data indicates that the PD PM hosts many receptors and receptor kinases, as well as lipid raft and tetraspanin enriched microdomain associated proteins, suggesting the hypothesis that the PD PM is specialized with respect to both composition and function. PD-located receptor proteins and receptor kinases are responsible for perception of microbe associated molecular patterns at PD and initiate signaling that mediates changes to PD flux. In addition, developmentally relevant receptor kinases have different interactions dependent upon whether located at the PD PM or the cellular PM. The implications of these findings are that receptor-mediated signaling in PD membranes differs from that in the cellular PM and, in light the identification of PD-located proteins associated with membrane microdomains and the role of membrane microdomains in analogous signaling processes in animals, suggests that the PD PM contains specialized signaling platforms.

Keywords: plasmodesmata, receptor kinase, receptor protein, lipid raft, tetraspanin enriched microdomain

INTRODUCTION – PLASMODESMAL STRUCTURE AND FUNCTION

Plant cells are connected to their neighbors via structural channels called plasmodesmata (PD), allowing the movement of molecules between cells and tissues. Molecular flux via PD is essential for many processes requiring intercellular communication and regulation of PD function can control the timing of signaling between cells in these contexts. PD are plasma membrane (PM) lined channels that cross the cell wall generating cytoplasmic and PM continuity between cells (Maule et al., 2012). The endoplasmic reticulum (ER) also passes from cell to cell via PD and the PD ER is known as the desmotubule. Trafficking from cell-to-cell has been observed to occur via the cytoplasmic channel (Oparka and Prior, 1988; Tucker et al., 1989), the desmotubule lumen (Barton et al., 2011), and the desmotubule membrane (Grabski et al., 1993; Martens et al., 2006; Guenoune-Gelbart et al., 2008), offering avenues for both soluble and lipid-based transport.

Our current understanding of PD function and the regulation of PD flux is limited. It has been established that PD allow the passage of molecules that are small enough to diffuse through the cytoplasmic sleeve and the current hypothesis is that dynamic regulation of the sleeve size dictates the size exclusion limit (SEL) for such molecules. This non-specific transport between cells is thought to be primarily regulated by the abundance of callose in the cell wall surrounding the necks of the channel (Zavaliev et al., 2011). Callose deposition pushes the PM inward to reduce the sleeve size and thus localized callose synthesis and hydrolysis regulates the flux of molecules through the channel (Maule et al., 2012). Specific and/or active transport between cells is possible for larger molecules such as transcription factors and viruses. In the case of the KNOX family transcription factors, this transport is mediated by a chaperonin which is required to unfold the transcription factor after passage through the PD channel, implicating protein folding as an essential component of the translocation mechanism (Xu et al., 2011). However, both KNOTTED1 (Lucas et al., 1995) and viral movement proteins (Wolf et al., 1989) can increase the PD SEL so it remains possible that this active and specific trafficking process also involves some alteration to PD structure.

Proteins located at PD are likely to have functions specific to the regulation and structure of PD. Recent work has identified several receptor kinases and receptor proteins that are specifically located, or function, at the PD PM (Thomas et al., 2008; Fernandez-Calvino et al., 2011; Lee et al., 2011; Faulkner et al., 2013; Stahl et al., 2013), in addition to callose synthases (Guseman et al., 2010; Vaten et al., 2011) and β-1,3-glucanases (Levy et al., 2007; Benitez-Alfonso et al., 2013) that mediate callose turnover specifically at PD. While the intermediate signaling steps are unknown, these results indicate that receptors exposed at the PD membrane perceive changes to the cellular environment and initiate a downstream cascade that ultimately regulates PD SEL and intercellular transport.

MEMBRANE MICRODOMAINS ARE PLATFORMS FOR RECEPTOR-MEDIATED SIGNALING IN ANIMAL CELLS

Subdivision of the PM into microdomains is required for membrane located processes in a variety of systems. In mammalian cells, membrane compartmentalization and microdomains define signaling processes that include B-cell and T-cell activation, apoptosis and insulin signaling. In these contexts, both lipid rafts and tetraspanin enriched microdomains (TEMs) alter signaling activity of specific receptors located in the PM. Lipid rafts and TEMs are microdomains of the PM that are differentiated by their lipid and protein composition. Lipid rafts are enriched in cholesterol and glycoshingolipids, and proteins such

as stomatin/prohibitin/flotillin/HflK/C (SPFH) domain proteins. In plants, lipid rafts are also defined by the presence of the plant-specific protein remorin (Jarsch and Ott, 2011). Like lipid rafts, TEMs are discrete areas of membrane but are defined by an enrichment of tetraspanin proteins. The difference in lipid and protein composition of membrane microdomains means they exhibit varying resistance to detergents. This allows the extraction of many raft and TEM components in detergent resistant membrane (DRM) fractions, although the biological accuracy of this fraction as corresponds to raft identity is a matter of some debate.

Tetraspanin enriched microdomains and lipid rafts act as platforms for receptor-mediated signaling in a number of contexts. In mammals, B-cell activation relies on the detection of an antigen by B-cell receptor (BCR) microclusters and BCR signaling occurs via the co-receptor CD19. CD19 is organized and immobilized in the membrane by the tetraspanin CD81 (Mattila et al., 2013). *Cd81*$^{-/-}$ mutant cells are deficient in downstream events such as effector phosphorylation (Mattila et al., 2013) illustrating that the membrane compartmentalization of CD19 and BCR is fundamental to the process of B-cell activation. TEMs also play a role in pattern recognition receptor (PRR) display and signaling. For example, the tetraspanin CD37 interacts with the β-glucan receptor Dectin-1 in antigen presenting cells and mediates induction of the defense-associated molecule interleukin-6 (Meyer-Wentrup et al., 2007).

In a similar fashion, lipid rafts provide an alternate membrane environment for receptor signaling. One such example is the regulation of apoptosis in mammalian cells by lipid raft localized signaling. Ligand-independent activation of apoptotic signaling by the tumor necrosis factor protein Fas involves oligomerization of the receptor in lipid rafts and subsequent recruitment of other components of the death-inducing signaling complex that triggers Caspase-8 activity and apoptotic signaling (George and Wu, 2012).

The common theme to the involvement of lipid rafts and TEMs in signaling is the spatial concentration (or separation) of signaling components. It seems likely that while there is little understanding of the primary functions of membrane microdomains in plant cells, lipid rafts and TEMs might facilitate signaling in a similar manner.

MEMBRANE MICRODOMAINS AT PD

Recent studies have identified that the protein composition of the PD PM differs from the cellular PM, with the PD PM containing a number of unique or enriched proteins (Thomas et al., 2008; Fernandez-Calvino et al., 2011; Stahl et al., 2013). Correspondingly, it is likely that the lipid composition of the PD PM also differs from the cellular PM and the possibility that the PD PM contains lipid rafts was raised by the localization of remorin to the PD PM (Raffaele et al., 2009). Remorin has a functional role in PD trafficking as the *Solanum tuberosum* Remorin (REM) 1.3 regulates trafficking of potato virus X (PVX) from cell-to-cell in tobacco (Raffaele et al., 2009). In *Arabidopsis*, *At*REM1.2 was identified in the PD proteome along with a number of SPFH domain proteins (Fernandez-Calvino et al., 2011), further suggesting the existence of lipid rafts in the PD PM.

Stomatin/prohibitin/flotillin/HflK/C domain proteins are found in lipid rafts in membranes in mammalian systems where they are associated with the compartmentalization of membranes, ion channel regulation, membrane trafficking and connection of membranes to the cytoskeleton (Browman et al., 2007). In plants, several SPFH proteins have been characterized and, as in mammalian systems, these proteins appear to have roles in the definition and activity of membrane domains. The *Arabidopsis* SPFH domain protein FLOTILLIN1 (FLOT1) was recently shown to function in clathrin-independent endocytosis, and immunogold labeling of the PM indicated that FLOT1 clustered in the PM in a manner consistent with its localization in microdomains (Li et al., 2012). Similarly, FLOT2 and FLOT4 are unevenly distributed in *Medicago* root cells (Haney and Long, 2010). The *Arabidopsis* HYPERSENSITIVE INDUCED REACTION PROTEINS (*At*HIR) are SPFH domain proteins and both *At*HIR1 and *At*HIR2 interact with the resistance protein RPS2. This interaction is required for defense responses triggered by RPS2 and occurs unevenly in the PM, suggestive of localization of activity in membrane sub-domains (Qi et al., 2011). The PD proteome contains the SPFH domain proteins *At*HIR1-4, FLOT1, a stomatin-like protein, an erlin-2-like protein and PROHIBITIN3 and 7 (Fernandez-Calvino et al., 2011). The association of SPFH domain proteins with lipid rafts, and their putative association with the PD PM, further allows the hypothesis that lipid rafts in the PD PM create PD-specific signaling platforms.

The identification of TETRASPANIN3 (TET3) in the PD proteome suggests that the PD PM also houses TEMs in addition to lipid rafts. TET3 was confirmed as a PD-located protein by subcellular localization of a TET3-YFP fusion (Fernandez-Calvino et al., 2011). There is scarce information relating to the abundance and function of TEMs in plant cells but recent characterization of the subcellular localization of a number of *Arabidopsis* tetraspanins provided some evidence that tetraspanins do associate with membrane microdomains in plant cells like in mammalian cells (Boavida et al., 2013). The localization pattern of TET5 was consistent with it being a PD-associated protein, but as yet no functional role in PD-specific membrane microdomains has been determined for either TET5 or TET3.

PROTEIN MICRODOMAINS AT PD

PLASMODESMATA LOCATED PROTEINS (PDLPs) were identified as an 8-member family of novel receptor proteins that are located at the PD PM (Thomas et al., 2008). PDLPs have two extracellular DUF26 domains, a transmembrane domain and a short cytoplasmic tail with the transmembrane domain of PDLP1 sufficient to convey PD targeting of a fluorescent reporter (Thomas et al., 2008). This suggests that PDLP1 is anchored at PD via its interaction with the membrane environment, either with another PD PM protein or with the membrane phospholipids present at the PD PM.

The specificity of PDLP localization indicates that the PD PM is differentiated from the cellular PM but in addition to this there is evidence that the PD PM is further subdivided into microdomains. While PDLPs were immunolocalized to the central PD PM, another PD PM associated protein, PLASMODESMATA CALLOSE BINDING1 (PDCB1, was immunolocalized to the PD

PM at the neck of the channel (Maule et al., 2011). Given the callose binding capacity of PDCB1 it is consistent that this protein is located at a site of callose deposition, but it should also be noted that PDCB1 is a glycophosphatidylinositol (GPI) anchored protein. GPI anchored proteins are tethered to the PM, preferentially at lipid rafts (Mayor and Riezman, 2004). Thus, considering the preference for localization of GPI anchored proteins within lipid rafts, it is possible that PD PM subdomains correspond with lipid rafts and/or TEMs.

RECEPTOR MEDIATED SIGNALING AT THE PD PM

Protein localization to and within the PD PM must hold functional significance for the mode of activity of proteins which show PD specificity. Accordingly, PD PM protein and membrane microdomains are likely to be fundamental to PD function. The observation that the lipid raft protein *St*REM1.3 has the capacity to directly bind the PVX TRIPLE GENE BLOCK1 protein and to regulate the trafficking of the virus from cell to cell (Raffaele et al., 2009) supports this hypothesis. It seems likely that PD PM microdomains contribute to the regulation of PD in multiple ways.

As described above, lipid rafts and TEMs in mammalian cells often function in receptor display and activation, providing a platform for specialized and localized signaling. This has particular relevance to receptor signaling in mobile immune cells, as illustrated by membrane microdomain involvement in B cell activation. At first glance, immune responses in plant cells have fundamental differences to those in animal cells as in plants each cell must be capable of initiating a response rather than being mediated by an army of specialized, mobile cells. However, in plant cells, early pathogen perception and defense responses are mediated by receptor kinases and receptor proteins exposed at the cell surface as in animal cells. These receptors trigger a medley of intracellular signaling events that launch defense responses.

Like for mammalian cells, lipid rafts have been associated with defense signaling in plant cells. For example, following treatment of *Arabidopsis* cell suspension cultures with the bacterial flagellin derivative flg22, a number or receptor kinases and other signaling proteins were enriched in DRM fractions (Keinath et al., 2010). These included the flagellin perceiving receptor kinase FLAGELLIN SENSING2 (FLS2) suggesting compartmentalization of this PRR in the PM. FLS2 also co-immunoprecipitates with the SPFH domain protein *At*HIR2 (Qi and Katagiri, 2012), providing further evidence that FLS2 activity associates with lipid rafts. This allows speculation that FLS2 activity, and that of other plant receptor kinases, is facilitated by recruitment to membrane microdomains like is seen in animal cells.

Recent work has identified PD PM located proteins that play a role in the regulation of intercellular flux during defense responses. *Arabidopsis* LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN 2 (*At*LYM2) is a PD-located, GPI-anchored receptor protein that perceives chitin and in response triggers PD closure (Faulkner et al., 2013). Significantly, *At*LYM2-mediated chitin perception and signaling occurs independently of other chitin-triggered responses such as an increase in reactive oxygen species (ROS burst) or mitogen activated protein kinase (MAPK) activation. Chitin-triggered ROS burst and MAPK activation are mediated by the receptor kinase CHITIN ELICITOR RECEPTOR KINASE1 (CERK1; Miya et al., 2007) which is present in the cellular PM and dimerizes in the presence of chitin (Liu et al., 2012). CERK1 is not required for chitin-triggered PD closure and therefore there is functional and spatial separation of chitin-triggered defense. Given that *At*LYM2 has a GPI anchor, and that the PD PM likely contains membrane microdomains, it is tempting to speculate that this difference in signaling location is facilitated by differential association of the relevant receptors with PD PM lipid rafts (**Figure 1**). CERK1 also forms a complex with the receptor proteins *At*LYM1 and *At*LYM3 for the perception of peptidoglycan (Willmann et al., 2011). Again, considering that *At*LYM1 is a GPI anchored protein, this complex formation might be mediated by recruitment of CERK1 to a different PM microdomain in the presence of peptidoglycan (**Figure 1**).

Recently, we showed that in addition to mediating flagellin triggered defense responses such as ROS burst and MAPK activation, FLS2 mediates flg22 induced closure of PD (Faulkner et al., 2013). A FLS2-GFP fusion is located at the PD PM as well as the cellular PM, suggesting that, like LYM2, it could trigger a site specific response. Upon binding of flg22, FLS2 forms a complex with another receptor kinase BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1) and this interaction is required for the initiation of FLS2 signaling cascades (Monaghan and Zipfel, 2012). It is not yet known whether PD closure is dependent on FLS2-BAK1 interaction but it is possible that either FLS2, or the FLS2/BAK1 complex, interacts with PD PM specific components that mediate FLS2-triggered PD closure. Given the association of FLS2 with *At*HIR2, and the identification of *At*HIR1-4 as putative PD PM proteins, it is possible that PD localization and signaling of FLS2 also occurs via interaction with lipid rafts at PD.

Plasmodesmata responses in the context of defense have also implicated the activity of PDLP5. *PDLP5* is upregulated in response to salicylic acid (SA) and PDLP5 regulates callose deposition to close PD in response to SA (Lee et al., 2011; Wang et al., 2013). Given that SA biosynthesis is an intracellular process, and that SA regulates *PDLP5* transcription, the role of PDLP5 as a receptor protein in this response is still unclear. Wang et al. (2013) proposed there might be a direct link between PDLP5 and callose synthases. Whether this link comes from direct complex formation between these proteins, or whether PDLP5 activity triggers a signal cascade that results in increased callose synthase activity remains to be determined.

Receptor mediated signaling at the PD PM is unlikely to be unique to defense responses. Two independent proteomic studies identified a number of receptor kinases that reside in the PD PM (Fernandez-Calvino et al., 2011; Jo et al., 2011) and thus it is probable that this membrane domain provides a platform for PD-relevant signaling initiated by a variety of triggers. Compelling evidence to support this comes from the observation that differential receptor-kinase complex formation occurs at the PD

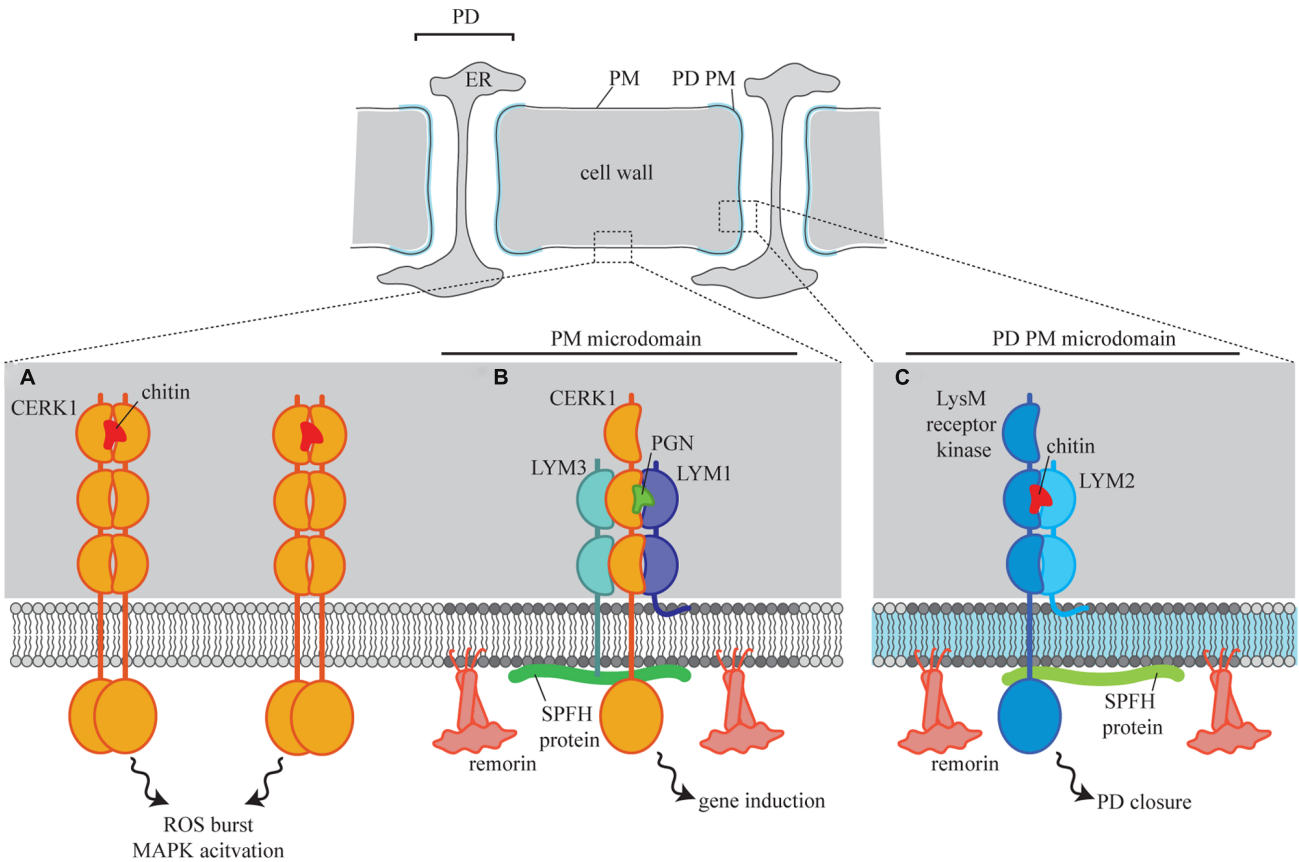

FIGURE 1 | A model for the spatial separation of LysM receptor signaling in plant cells. In the cellular PM CERK1 perceives chitin and triggers a number of defense responses intracellularly **(A)**. Chitin perception at the PD PM **(C)** requires the GPI-anchored receptor protein LYM2, via interaction with an unknown LysM receptor kinase. Remorin and SPFH proteins in the PD PM define lipid rafts which act as platforms for this signaling complex. In the cellular PM CERK1 is recruited to lipid rafts where it interacts with LYM1 and LYM3 to form a peptidoglycan (PGN) perception complex **(B)**.

PM during the definition of root stemness (Stahl et al., 2013). The receptor kinases CLAVATA1 (CLV1) and ARABIDOPSIS CRINKLY4 (ACR4) are involved in maintenance of the root meristem and can form both homo- and heteromeric protein complexes. While both receptors are present in the PM, ACR4 accumulates at the PD PM relative to the cellular PM. FRET-FLIM experiments allowed the authors to propose that the cellular PM contains CLV1-ACR4 heterodimers and ACR4-ACR4 homodimers while at the PD higher order homo- and heteromeric complexes form due to the higher concentration of ACR4 (Stahl et al., 2013). Cell fate specificity and developmental processes have been shown to depend on the intercellular movement of proteins such as transcription factors in several tissues. The specific PD-associated function of ACR4 and CLV1 has not yet been determined but presumably the higher order complexes of ACR4 and CLV1 in the PD PM mediate PD specific signaling that regulates the PD aperture and the movement of a non-cell autonomous signal that defines root stemness. It is again possible that the concentration of ACR4 in the PD PM, and the formation of a differential signaling platform, is a consequence of specific recruitment of ACR4 to a PD PM microdomain defined by the lipid environment.

CONCLUSION

There is a significant body of evidence that suggests both lipid rafts and TEMs provide signaling platforms in plant cells. Recent advances have highlighted the specificity of a number of PD PM located receptor proteins and receptor kinases that have PD specific functions. When combined with the identification of a number of lipid raft and TEM associated proteins in the PD PM we can begin to build a model in which PD specific receptors are localized and activated via recruitment to PD PM microdomains. Future work to characterize the composition of the PD PM and the signaling cascades triggered by the resident proteins will elucidate mechanisms of PD function and regulation, allowing a more in depth understanding of intercellular communication and co-ordination.

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Imaging lipid domains in cell membranes: the advent of super-resolution fluorescence microscopy

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The lipid bilayer of model membranes, liposomes reconstituted from cell lipids, and plasma membrane vesicles and spheres can separate into two distinct liquid phases to yield lipid domains with liquid-ordered and liquid-disordered properties. These observations are the basis of the lipid raft hypothesis that postulates the existence of cholesterol-enriched ordered-phase lipid domains in cell membranes that could regulate protein mobility, localization and interaction. Here we review the evidence that nano-scaled lipid complexes and meso-scaled lipid domains exist in cell membranes and how new fluorescence microscopy techniques that overcome the diffraction limit provide new insights into lipid organization in cell membranes.

Keywords: lipid rafts, membrane microdomains, super-resolution, fluorescence, cell membranes

INTRODUCTION

In the fluid mosaic model (Singer and Nicolson, 1972), the lipid bilayer was originally viewed as a simple 2D fluid in which embedded membrane proteins are able to diffuse freely in the lateral dimension. Many observations however, showed that lipids and membrane proteins are not homogeneously distributed in the plasma membrane. As early as 1987 for example, it was shown that in MDCK cells, sphingolipids first accumulate in the Golgi and are then transported to the apical surface where they are unable to diffuse past tight junctions at cell−cell contact sites (van Meer et al., 1987). If the lipid distribution of the plasma membrane is indeed regulated and non-random, this suggests that biophysical processes exist in cells that cause a lateral organization within the membrane and/or active mechanisms have evolved by which cells sort protein and lipids. It is highly likely that such lateral organization is exploited for specific cell functions.

In 1997, Simons and Ikonen proposed the lipid raft hypothesis in which the phase behavior of different lipid species is exploited to create lateral heterogeneity in the plasma membrane (Parton and Simons, 1995; Simons and Ikonen, 1997). According to this hypothesis, the liquid-disordered phase, formed mainly from unsaturated phospholipids, would coexist in the plasma membrane with a liquid-ordered phases formed from saturated phospholipids and sphingolipids in the presence of cholesterol, which exists in the plasma membrane at concentrations of roughly 30 mole percent. In the ordered phase, a higher degree of conformational order is imposed on the acyl tails of lipids by the rigid ring structure of cholesterol. This results in an increase in the thickness of the lipid bilayer and tighter lipid packing although unlike the gel phase (consisting of saturated lipids in the absence of cholesterol), liquid-ordered bilayer lipids remain laterally mobile. In this model therefore, the plasma membrane is viewed as a "sea" of disordered phase lipids containing stable, ordered phase "islands" or "rafts" enriched in saturated lipids, sphingolipids and cholesterol.

It was then hypothesized that specific membrane proteins would have a high affinity for one phase, thereby partitioning into this phase and being laterally sorted. This would allow lipid rafts to serve as signaling platforms, concentrating some proteins to facilitate their interaction while excluding others (Levental et al., 2010). The specific proteins that would be concentrated in such domains would depend on the type of membrane targeting sequence (Brown, 2006). For example, transmembrane proteins with a longer transmembrane domain that closely matches the increased thickness of the ordered phase bilayer would show affinity for these domains, as this would minimize the hydrophobic mismatch energy. Similarly, proteins that are post-translationally modified with long, saturated acyl chains would show affinity for ordered domains in the same way as saturated bilayer lipids themselves show ordered phase affinity.

While the coexistence of micron-scale, resolvable ordered and disordered phase lipid domains was readily observed in model membranes using fluorescence microscopy and phase-partitioning membrane probes (Simons and Vaz, 2004), no such structures have been observed in cell membranes. Although biochemical techniques such as detergent extraction continued to be used (London and Brown, 2000; Shogomori and Brown, 2003), the lack of direct imaging caused the lipid raft hypothesis to become controversial (Munro, 2003; Glebov and Nichols, 2004; Hancock, 2006) and the definition of a lipid raft has evolved over the years. Originally, lipid rafts were defined as "preferential packing of sphingolipids and cholesterol in moving platforms, or rafts, onto which specific proteins attach within the bilayer" (Simons and Ikonen, 1997). The lack of direct visualization resulted in an emphasis on the sub-diffraction-limited size of the domains such that they were described as being a

"molecular complex in the membrane [that] consists of at least 3 molecules that includes a molecule with a saturated alkyl chain or a cholesterol molecule that plays a critical role in the formation of the complex itself" (Kusumi et al., 2004). An example of a yet later definition emphasizes the dynamic nature of the domains defining rafts as "small (10–200 nm), heterogeneous, highly dynamic, sterol and sphingolipids-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein or protein–lipid interactions" (Pike, 2006). The frequent modifications of the lipid raft hypothesis have questioned its validity but the hypothesis was undoubtedly the snowball that triggered new thinking and the emergence of new membrane models. Its emphasis on lipids was the motivation to develop new tools for lipid research. However, it should be kept in mind that organelle and plasma membranes of cells contain an extremely high protein density (Takamori et al., 2006; Dupuy and Engelman, 2008). Therefore one should not simply envisage cell membranes as systems where proteins floating in a "sea" of lipids. Instead, the membrane must be treated as a "lipid–protein composite" in which a very high density of transmembrane domains may impose order on nearby lipids complimenting lipid domains organizing proteins (Jacobson et al., 2007).

In some of these definitions, a substantial cohesion length that is a characteristic of a phase in model membranes is no longer included so that no distinction between nano-scaled complexes and meso-scaled domains are made. This lack of distinction may make it difficult to translate findings from pure lipid bilayers to complex cell membranes because the lack of microscopically visible lipid domains in cells is not proof of the absence of lipid rafts. Whether complexes of a few molecules could indeed be called a phase is biophysically controversial and for this reason, we continue to distinguish between multi-molecular complexes and meso-scale domains. Although this limit is arbitrary, meso-scaled domains should be above 20 nm in size and thus contain several thousand lipids (Pralle et al., 2000).

Defining lipid phases is not an issue in model membranes and thus lipid phase have been precisely mapped in such systems resulting in phase diagrams that show the phase behavior at different lipid compositions (Bezlyepkina et al., 2013). It is now recognized that the composition of the plasma membrane of cells in most cell types lies close to the critical composition for the liquid-ordered, liquid-disordered phase transition of lipid mixtures containing pure unsaturated phosphatidylcholine, sphingomyelin, and cholesterol (Lingwood et al., 2008). This may be a mechanism by which small changes in composition or environmental factors can cause large changes in organization. This was recently observed when resolvable sterol-enriched domains were found to form in the vacuole membrane of yeast cells in response to physiological changes, such as pH (Toulmay and Prinz, 2013).

Despite the lack of direct observation of lipid phases in intact and live cells, ordered-phase membrane domains are thought to play a role in a wide range of cellular processes, mainly in signaling at the plasma membrane and the selective trafficking of lipid components. We have used polarity sensitive membrane dyes, such as Laurdan, to quantify membrane order *ex vivo* and *in vivo* in intact zebrafish embryos (Owen et al., 2010a, 2012b). Even though fluidity differences in the plasma membrane are readily observed between cell types and cellular conditions, clear evidence of lipid phases in cell membrane could not be obtained with diffraction-limited imaging (Gaus et al., 2003). However, correlations between membrane order and cell functions were established. For example during T cell activation, high membrane order has been shown to be required for the correct localization of membrane-associated proteins and efficient T cell signaling (Rentero et al., 2008; Ventimiglia and Alonso, 2013). Membranes of high order were localized at the periphery of T cell synapse which is associated with actin and adhesion proteins, indicating a link between lipid organization and the actin cytoskeleton (Owen et al., 2010b). In addition, sub-synaptic vesicles with a high membrane order have also been observed, which may be important in the trafficking of specific T cell components, such as the raft-associated adaptor protein linker for activation of T cells (LAT; Williamson et al., 2011). Lipid rafts have similarly been implicated in various aspects of B cell signaling (Gupta and DeFranco, 2007). Other roles for highly ordered membrane domains include focal adhesions (Gaus et al., 2006) and cell migration (Gomez-Mouton et al., 2004), virus entry and budding (Mañes et al., 2000; Carrasco et al., 2004; Khurana et al., 2007; Lorizate et al., 2009), autoimmune disease (Jury et al., 2007; Miguel et al., 2011), the blood-brain barrier (Dodelet-Devillers et al., 2009), hormone signaling (Márquez et al., 2006; Yang et al., 2010) and in the trafficking of lipids in polarized cells (van Meer et al., 1987).

Most of the work to define lipid rafts experimentally has been conducted in artificial membranes, mammalian cells (both primary and cell lines) and yeast. Progress has also been made in analyzing membrane domains in plant cells. This has included the observation that detergent resistant membranes extracted from plant cell membranes (Peskan et al., 2000) which were found to be enriched in sterols and sphingolipids, similar to mammalian cells (Borner et al., 2005). This finding was later the subject of several reviews (Martin et al., 2005; Grennan, 2007). The similarity of plasma membrane order properties between plant and mammalian cells was reinforced by the observation that the membrane fluidity of bacteria, plant, mammalian and fungal membrane properties may display convergent evolution to a similar level regardless of membrane composition between species (Kaiser et al., 2011).

NEW INSIGHTS FROM SUPER-RESOLUTION IMAGING

Much of the controversy surrounding lipid rafts developed as a result of their supposed small size which made them impossible to image using standard fluorescence microscopy approaches. This is because the resolution of a conventional fluorescence microscope is limited by diffraction to above 200 nm. However, in recent years, three families of techniques have emerged which all break the diffraction barrier and allow imaging of cellular structures far below the conventional 200 nm limit. These methodologies are structured illumination microscopy (SIM), stimulated emission depletion microscopy (STED) and photoactivated localization microscopy (PALM). Many of these techniques and now starting to be applied to imaging plant cells (Fitzgibbon et al., 2010; Kleine-Vehn et al., 2011). The major advantages and disadvantages

Table 1 | Summary of super-resolution imaging techniques to probe membrane organization below the diffraction limit.

	PALM/STORM	STED	SIM	NSOM
Lateral resolution	20–30 nm	60–100 nm	100–120 nm	20–30 nm
Image speed	Minutes	Seconds	Seconds	Seconds
Image and sample geometry	2D or 3D image of fluorophores close to coverslip	2D image at any focal plane	3D image over entire cell	Only surface proteins
Equipment complexity	Simple	Complex	Intermediate	Complex
Analysis complexity	Complex	Simple	Intermediate	Intermediate

of the techniques discussed here are summed up in **Table 1** for typical biological samples.

In SIM, the sample is illuminated with a grid pattern, which is then shifted while multiple images are acquired. A super-resolution image is then calculated computationally from the data. SIM can achieve resolutions of around 100 nm in lateral direction, can perform 3D imaging in live cells (although this is still technically challenging) and uses conventional fluorophores (Gustafsson, 2000; Kner et al., 2009; Shao et al., 2011).

Stimulated emission depletion microscopy uses a doughnut-shaped depletion laser beam to de-excite fluorophores at the periphery of a confocal excitation spot. This narrows the size of the spot thereby increasing the resolution. Depending on what laser powers the sample can tolerate from the depletion beam, resolutions of 50–100 nm laterally are typically possible in biological samples. The technique is built on a conventional laser-scanning microscope and has been applied to live cell imaging (Hell and Wichmann, 1994; Hein et al., 2008; Vicidomini et al., 2011).

Photoactivated localization microscopy and related techniques image and localize individual fluorophores, which typically results in localization precisions of individual molecules of around 20–30 nm. While the technique has long acquisition times and is generally a 2D technique based on total internal reflection fluorescence (TIRF) illumination, progress is being made in establishing 3D PALM as well as higher-speed imaging for live cell analysis (Betzig et al., 2006; Rust et al., 2006; Klein et al., 2011).

These methods have delivered previously unattainable data on membrane lipid domains and any proteins have been shown to be clustered within the plane of the membrane using super-resolution methods which otherwise appear homogeneous in conventional resolution systems (Owen et al., 2012a). PALM (**Figure 1**) can be used to map the localization of raft and non-raft targeted fluorescent fusion proteins and a quantitative analysis can distinguish protein clusters from random distributions, frequently identifying clusters on scale of 50–100 nm (Owen et al., 2010c, 2012a,c; Sengupta et al., 2011; Sengupta and Lippincott-Schwartz, 2012). One of the earliest single-molecule super-resolution data demonstrated the nano-scale clustering of Hemagglutinin (Hess et al., 2007), which is thought to cluster in lipid rafts (Takeda et al., 2003) and was more recently shown to cluster in an actin-dependent manner (Gudheti et al., 2013). Sengupta et al. (2011) used PALM and pair-correlation analysis to show that glycosylphosphatidylinositol (GPI)-anchored proteins formed nano-clusters there were sensitive to the cellular levels of cholesterol and sphingomyelin and cross-correlated with actin after antibody cross-linking (Sengupta et al., 2011). Similarly, we used a distribution analysis based on Ripley K-function to quantify the non-random distribution of membrane proteins (Owen et al., 2010c) and identified for example that the conformational states of the kinase Lck can regulate clustering, thereby linking intramolecular arrangement to intermolecular patterning (Rossy et al., 2013). However, it is not clear to which extent protein clustering reflects the underlying lipid organization. In unpublished data, we found that even weak protein–protein interactions induced by the fluorescent protein mEOS2 could cause clustering of raft-favoring and non-raft lipid anchors independently of the membrane fluidity. This suggests that protein interactions could easily override that partitioning preference of a protein into lipid phases. Hence localizing proteins may not be sufficient to map the distribution and geometry of lipid domains in cell membranes. To our knowledge, there are currently no lipid probes available that could be used to map lipid domains in cell membranes with PALM. Since the partitioning of fluorescent lipids into liquid-order and liquid-disordered phases differs markedly whether phases in model membranes or cell-derived membrane vesicles are examined (Sezgin et al., 2012), one can also not solely rely on the distribution of different lipids to map lipid domains. Hence more sophisticated lipid probes are needed to utilize the localization power of PALM to image lipid domains.

Excitingly, super-resolution microscopy also has the ability to generate new information on molecular dynamics. STED has been combined with fluorescence correlation spectroscopy (FCS) – a method for determining molecular diffusion coefficients based on fluorescence fluctuation analysis (**Figure 2**). This allows dynamics to be analyzed on sub-resolution length scales similar to what has been achieved previously with near-field scanning optical microscopy (NSOM) based techniques (Vobornik et al., 2008), but with a controllable spot size. Using STED FCS in cells, it was shown that sphingolipids and glycophosphatidylinositols (two putative raft markers) become transiently arrested in the plasma membrane whereas phosphoglycerolipids (non-raft molecules) do not. This trapping was cholesterol dependent, occurred in ∼20 nm areas and lasted on the order of tens of milliseconds (Eggeling et al., 2009). A similar observation using STED FCS was also shown for cytoskeletal-dependent transient trapping (Mueller et al., 2011). Interestingly, a modified saturated phosphoethanolamine could be used to map liquid ordered domains in model systems below

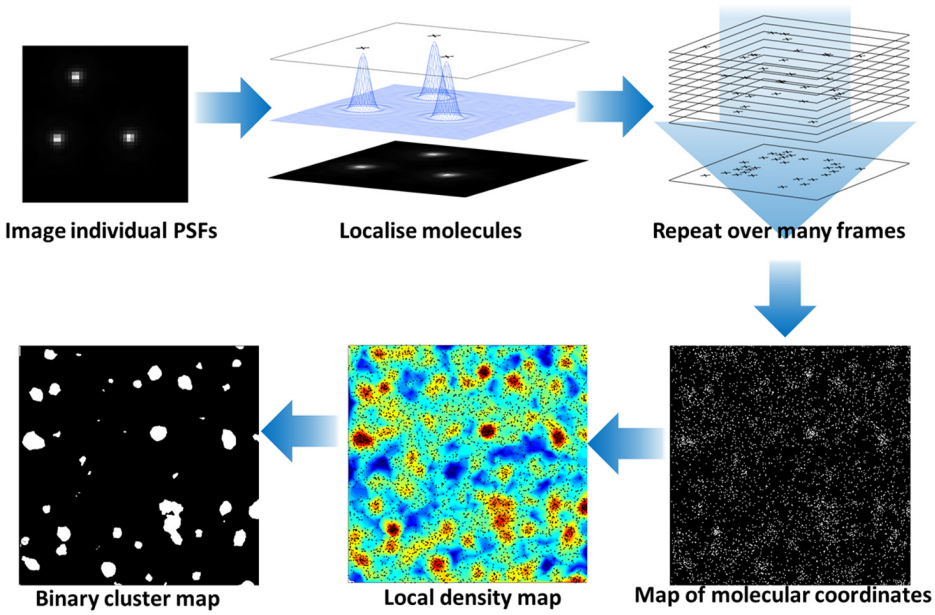

FIGURE 1 | Photoactivated localization microscopy analysis of protein clustering at the cell surface. Photo-activation or stable dark states of fluorophores are exploited to limit the number of fluorescent molecules in each image frame. The fluorescence of individual molecules are captured with a camera and the center of the point-spread function (PSF) calculated to localize the molecules with nanometer precision. During the imaging processes, the fluorescence molecules are bleached so that the combination of photo-activation and photo-bleaching gives the appearance that molecules "blink" during the acquistion. Over successive frames, an image of all fluorescent molecular positions is built up. The molecular coordinates can be used to generate an image and be quantitatively analyzed to reveal the local density of fluorescent molecules (here based on Ripley K-function before and after application of a threshold) and hence clusters of proteins at the plasma membrane identified. For details on the cluster analysis, please see Williamson et al. (2011).

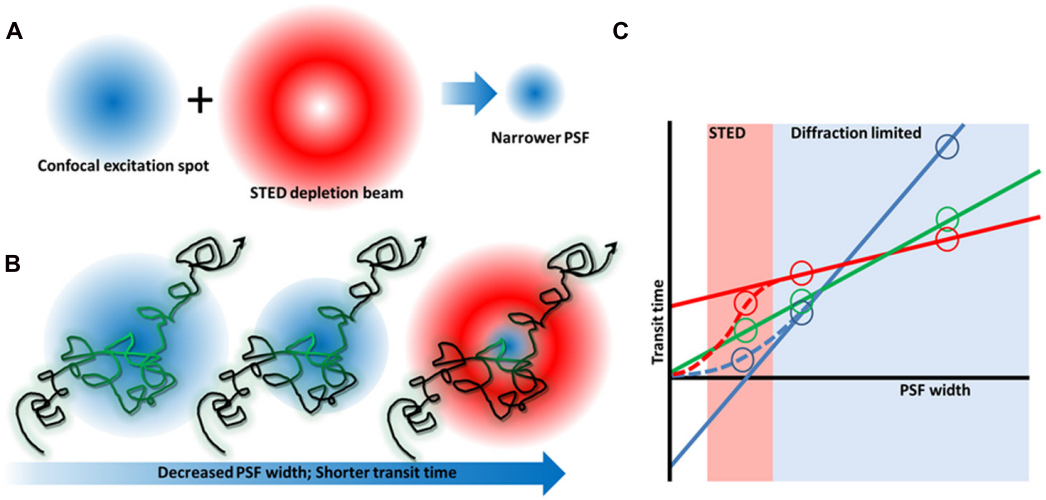

FIGURE 2 | Stimulated emission depletion microscopy microscopy and FCS diffusion laws. (A) By combining a red-shifted, "doughnut"-shaped depletion beam with a confocal laser beam, the excitation spot and hence PSF can be narrowed. **(B)** FCS measures the time fluorescent molecules take to diffuse through the focus of a stationary beam. By decreasing the PSF width, the transit time becomes shorter so that even short lived complexes can be detected whose existence is canceled out when a larger observation area is used (Wawrezinieck et al., 2005). **(C)** By varying the PSF width, a plot of transit time vs spot size can be generated that reveals free diffusion (green), or membrane heterogeneity caused by an actin meshwork (blue), or membrane domains (red).

the diffraction limit (Honigmann et al., 2013) but showed no trapping in cells (C. Eggeling, personal communication). Collectively, the STED FCS data in cell membranes point more toward lipid complexes that are short lived, rather than lipid domains that may be positionally and temporally stable. Chemical modification of lipids may affect their dynamics and complex formation and hence like PALM, this super-resolution technique also depends on the availability of well-characterized probes for lipid research.

We would like to point out that near field scanning-type imaging approaches such as NSOM can analyze membrane organization at smaller length scales than are possible using conventional microscopy. In NSOM, the effects of diffraction are circumvented by placing the detector (typically a fiber) very close (much less that the wavelength of light) to the sample, detecting the emitted fluorescence and then raster-scanning to build up an image which can result in lateral resolutions of less than 10 nm. For example, this technique has been used to show that GPI-anchored proteins, commonly used as lipid raft markers, are arranged in nano-scale clusters on the surface of immune cells (van Zanten et al., 2009). These "hotspots" were found to be essential for integrin-based cell adhesion. In T cells, NSOM was used to detect clusters of CD3, CD4, and CD8 membrane proteins on the cell surface on nano- and meso- length-scales (Zhong et al., 2009). In a similar study, NSOM showed that the nanoscale organization of proteins and lipids in T cells was temperature dependent (Chen et al., 2009), consistent with the classical lipid raft hypothesis and the observation of cold-induced activation of T cells (Magee et al., 2005). Similar to STED, NSOM has also been paired with FCS to reveal differences in anomalous diffusion of phosphoethanolamine and sphingomyelin (Manzo et al., 2011).

Although not a super-resolution technique, we recently used fluorescence lifetime imaging microscopy (FLIM) to gain insights into lipid organization in cell membranes below the diffraction limit. This was possible because we used an unbiased unmixing approach, the so-called phasor apporach, to map the spectral signatures of Laurdan in each pixel. We could show that Laurdan in the plasma membrane of HeLa show is not a homogenous phase of intermediate order but a mixture of ordered and disordered domains. By using the pure lipid mixtures of 70:30 sphingomyelin:cholesterol and 100% dioleoylphosphatidylcholine as reference points for liquid-ordered and liquid-disordered phases, we estimated that ∼76% of the plasma membrane is covered with ordered phases. This approach could not tell us whether Laurdan with an ordered FLIM signature comes from a continuous phase or from many domains and complexes with a large variation in sizes, simply because the data acquisition was still diffraction limited. One should also take into consideration that the liquid-ordered and liquid-disordered membranes in cells may have significantly different properties than the pure lipid mixtures that we used as reference data. It was for example shown that the difference in membrane order between phase-separated ordered and disordered domains in plasma membrane vesicles was much smaller than the differences observed in model membranes (Kaiser et al., 2009). However, combining environmentally sensitive probes with super-resolution technique may allow us for the first time to directly measure the bilayer properties of cell membranes. Unfortunately with Laurdan, this is not possible since it neither has a stable dark state for PALM nor is it STED-compatible due its fast photo-bleaching. But with more environmentally sensitive probes being developed (Bacia et al., 2004), we remain hopeful to one day characterize and map lipid complexes and domains in cell membranes.

THE EFFECT OF THE ACTIN CYTOSKELETON

One of the biggest changes to our current understanding of membrane heterogeneity has been an elevation of the role of the cytoskeleton (Edidin, 2006). The cortical actin mesh has frequently been a target for new super-resolution based imaging methods, for example 3D PALM (Xu et al., 2012, 2013), SIM (Brown et al., 2011) and STED (Rak et al., 2011). The density and dynamics of the cortical actin network make this structure a defining feature of cell membranes.

Firstly, the cytoskeleton can directly influence the diffusion and clustering of membrane proteins. The main theory here is the "picket fence" or "hop diffusion" model first developed by Kusumi et al. (2005). This theory holds that the cortical actin cytoskeleton forms a meshwork under the plasma membrane to which it is anchored by actin and bilayer-associated proteins. Molecules diffusing in the plasma membrane encounter these proteins as barriers causing them to be trapped in so called "transient confinement zones." From time-to-time, lipids and proteins may be able to "hop" over these barriers thereby becoming trapped in a new zone (Fujiwara et al., 2002; Kusumi et al., 2005; Morone et al., 2006). Such compartmentalization would be a size-dependent process where proteins containing a large intracellular domain or transmembrane proteins would experience a greater barrier to diffusion caused by the underlying mesh (Heinemann et al., 2013). Where membrane proteins are linked to the dynamic cortical actin mesh, it has been shown that fluctuations in the cytoskeleton can cause transient focusing (clustering) of the plasma membrane proteins (Chaudhuri et al., 2011) as the actin grid spacing fluctuates. Actin-tethered membrane proteins may also form clusters *via* short, dynamic actin fibers aligning assembling into aster formations (Gowrishankar et al., 2012; **Figure 3**).

It has recently been shown that many membrane proteins have their diffusion and distributions regulated by cortical actin (Gudheti et al., 2013; Mattila et al., 2013). While much of the early work on hop diffusion was performed using extremely high speed single molecule and single particle tracking to map confinement zones, this area has also proves fertile for the use of variable spot-size FCS (**Figure 2**). By performing FCS experiments over a range of size scales, it is possible to infer information on the underlying, sub-resolution organization without requiring more complex super-resolution hardware. These so called "FCS diffusion laws" make it possible to determine whether it is transient confinement zones or membrane lipid domains that exert the greatest influence on diffusion within the bilayer (Lenne et al., 2006; Lasserre et al., 2008). For example, the lipid ganglioside GM1, one of the archetypal lipid raft components is influenced mainly by lipid domains, whereas the large transmembrane protein Transferrin-1 has strong interactions with the cytoskeletal meshwork (Wawrezinieck et al., 2005).

It may also be the case that the cytoskeleton causes an increase in membrane lipid order (the abundance of the liquid-ordered

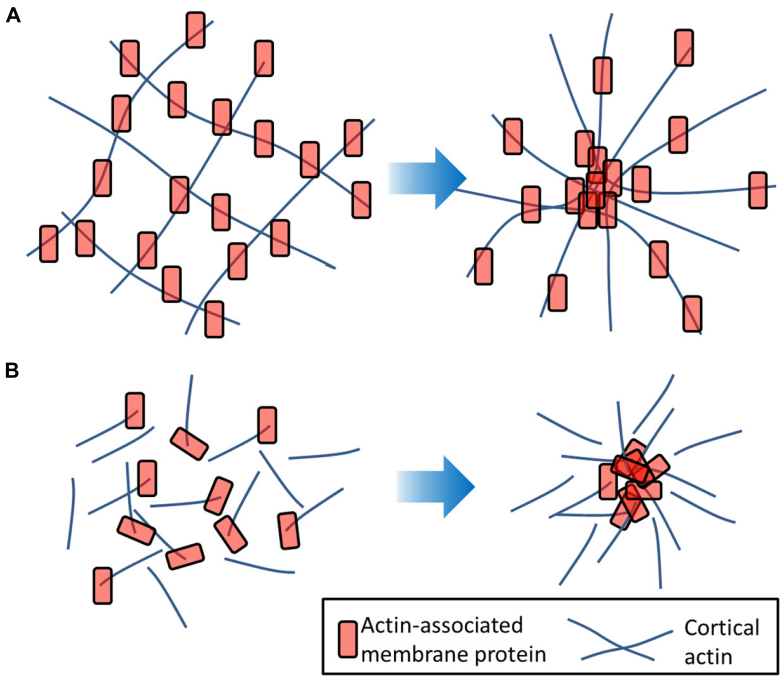

FIGURE 3 | Actin-induced clustering of membrane proteins. **(A)** Membrane molecules (red) which are tethered to the underlying actin filaments (blue) may undergo transient clustering as the actin mesh flexes. **(B)** Proteins anchored to short, dynamic actin strands may undergo clustering in response to actin aster formation that requires a motor such as myosin and is therefore an energy-dependent process.

phase) and therefore influences diffusion and distributions indirectly by regulating the bilayer phase behavior. Blocking actin polymerization using latrunculin causes a decrease in membrane order observed with the environmentally sensitive membrane probe di-4-ANEPPDHQ (Jin et al., 2006). Membrane order was also low in plasma membrane blebs in which the bilayer had been detached from the underlying cytoskeleton. Stabilization of the actin meshwork using jasplakinolide had the opposite effect and caused an increase in membrane order (Dinic et al., 2013).

It has been hypothesized that the cytoskeleton may cause "pinning" of local membranes in an ordered state, which then act as nucleation sites for the development of ordered-phase domains. Using computer modeling, it was demonstrated that if such pinning took place in a membrane that was very close to the critical composition for fluid – fluid phase coexistence, small critical fluctuations could cause many of the properties attributed to rafts, such as their small size and transient nature (Machta et al., 2011). Moreover, these critical fluctuations caused the formation of transient channels within the plans of the membrane, which could potentially regulate the interactions of membrane proteins over multiple length scales. This fits with the recent observation that the plasma membrane of cells contains a much higher coverage of the ordered phase than previously thought (Owen et al., 2012c) so that interactions may be controlled by which phase is the percolating "sea" phase and which phase represents the "islands" (**Figure 4**). While we have do direct evidence that phase geometry frequently change in cell membranes, coverage of 30–70% of either phase afford the possibility that protein interactions occur during the meso-scaled remodeling of phase geometries.

The high level of ordered-phase coverage could be the result of the extremely high density of membrane proteins (estimated at 23% protein coverage for the red blood cell membrane; Dupuy and Engelman, 2008) in the bilayer which impose order on the surrounding 1–2 shells of lipids adjacent to the protein (Jacobson et al., 2007). Such is the typical density of transmembrane domains (Takamori et al., 2006) that the membrane can be considered a lipid-protein composite and therefore the lipid properties may be dominated by transmembrane proteins (Jacobson et al., 2007). The switching of the "percolating" to the "island" phase could allow large changes in organization in response to very small changes in the physical environment (Lingwood et al., 2008). In this model, the partitioning of proteins into distinct phases is no longer the controller of specific interactions that then take place under static conditions. Instead, the switching of the percolating phase would allow selective mixing of components and hence would provide dynamic regulation. Such changing connectivity of different membrane domains and sub-regions has previously been observed by NSOM microscopy and in silico simulations (van Zanten et al., 2010). If such percolating phase switching indeed takes place, the dynamic properties of the cortical actin undoubtedly play a role.

CONCLUSION

Newly developed imaging techniques which allow super-resolution are dramatically increasing our understanding of the

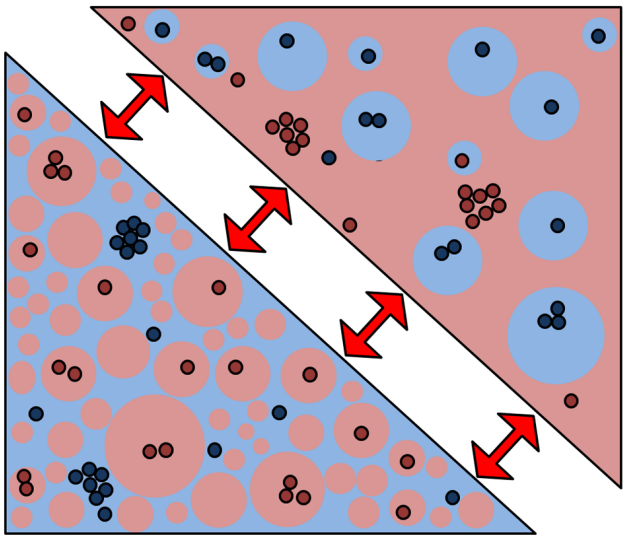

FIGURE 4 | A new membrane model based on percolating phase switching. A new membrane model emerged from our data that indicates that the plasma membrane is indeed a mixture of ordered (red) and disordered (blue) phases where the ordered phase is the majority. Without changing the fraction, protein interactions may be regulated when the geometry of the phases switches (red arrows) from the percolating to the "island" phase. In so-called critical fluctuations, even small perturbations can trigger large-scale changes such as phase geometry.

complexity of cell membrane organization. While the basic principles of the original lipid raft hypothesis – ordered membranes based on cholesterol and saturated lipids – may remain, more details have already emerged that cause the distinction between lipid domains into which certain proteins may partition and lipid complexes that may contain multiple proteins. Other forces at work include direct protein–protein interactions, ordering of shell lipids by protein transmembrane domains, critical transient lipid composition fluctuations and a complex interplay between the bilayer and the underlying actin cytoskeletal meshwork. This structure may influence the distribution of membrane proteins directly or *via* its effects on membrane lipid order. Further technological advances, particularly the development of functional probes that report on the membrane environment are undoubtedly needed to answer many of the outstanding questions of the organizational hierarchy of cellular membranes. What started as one hypothesis that brought lipids back into the focus has now evolved into a number of competing membrane models that are not mutually exclusive. Excitingly, as we understand more of how cell membranes are organized, we also gain deeper insight into functional processes such as receptor signaling and cargo-driven endocytosis.

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Membrane microdomains: from seeing to understanding

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The plasma membrane is a composite material, which forms a semi-permeable barrier and an interface for communication between the intracellular and extracellular environments. While the existence of membrane microdomains with nanoscale organization has been proved by the application of numerous biochemical and physical methods, direct observation of these heterogeneities using optical microscopy has remained challenging for decades, partly due to the optical diffraction limit, which restricts the resolution to ∼200 nm. During the past years, new optical methods which circumvent this fundamental limit have emerged. Not only do these techniques allow direct visualization, but also quantitative characterization of nanoscopic structures. We discuss how these emerging optical methods have refined our knowledge of membrane microdomains and how they may shed light on the basic principles of the mesoscopic membrane organization.

Keywords: suppersolution, plasma membrane microdomains, quantitative imaging, multiscale organization, protein clusters

INTRODUCTION

Cell functions strongly rely on its capacity to interact with neighboring cells and extracellular environment. Vital interactions such as metabolic material exchange and biochemical signaling are mediated by the plasma membrane, a semi-permeable barrier which covers the cell surface and separates a cell from its surrounding environment. While the seminal observations of a cell membrane dates back to the early 17th century with the first optical microscopy images of fly eyes and cork tissues (Hooke, 1665), the complexity of the cell membrane is currently being deciphered. The landmark model of the plasma membrane, called the "mosaic fluid model" (Singer and Nicolson, 1972) hypothesizes that plasma membrane is composed of a lipid bilayer housing different freely diffusing membrane proteins. This model implies a homogenous organization of membrane materials, yet did not represent the complete picture. In fact, the plasma membrane is highly asymmetric, with differences in lipid and protein compositions between the inner and the outer leaflet of the bilayer (Rothman and Lenard, 1977; Daleke, 2003). Moreover, the plasma membrane is laterally compartmentalized. Different lipid components can segregate into functionalized microdomains, often referred as lipid rafts (Simons and Ikonen, 1997). Lipid rafts were initially defined as liquid ordered domains enriched in sphingolipid and sterol, and surrounded by a liquid disordered phase (Simons and Ikonen, 1997; Simons and Van Meer, 1988). They can selectively recruit different membrane proteins, such as the glycosyl phosphoinositol (GPI)-anchored proteins (Simons and Van Meer, 1988; Simons and Ikonen, 1997), viral glyco-protein (e.g., Haemagglutinin and neuraminidase; Zhang et al., 2000), thus form protein–lipid microdomains. As cell protein concentration is high [e.g., 23% of the cell surface for red blood cell (Dupuy and Engelman, 2008)], protein–protein interactions can also yield the formation of microdomains, often referred as clusters [e.g., activated Ras-kinases (Prior et al., 2003), immune protein CD2, LAT (Douglass and Vale, 2005; Lin and Shaw, 2005)]. Transmembrane protein clusters can interact with intracellular cytoskeleton network, which can transiently trap proteins during diffusion and act as a "membrane-skeleton fence" (Kusumi et al., 1993, 2005; Sako and Kusumi, 1994). Thus, microdomains and clusters are shared features for lipids and proteins in membranes and are often considered as a "membrane-organizing principle" (Lingwood and Simons, 2010; Mongrand et al., 2010).

Membrane microdomains have been proposed to be essential for different cellular functions. Lipid rafts are proposed to facilitate the apical sorting of different membrane proteins in polarized cell (e.g., epithelial cells). The transport of vesicles enriched with raft markers such as cholesterol and sphingolipids has been shown to be polarized toward the apical surface (Simons and Van Meer, 1988). While some apical proteins have been proven to be preferentially associated with lipid rafts, basal proteins are not. It was suggested that lipid rafts could selectively recruit apical proteins and function as determinant apical landmarks for protein transport during biosynthesis (Simons and Ikonen, 1997). Furthermore, microdomains are important for biochemical reactions of membrane proteins. Compartmentalization by microdomains and clusters may provide a local optimal environment to facilitate the speed and efficiency of these reactions (Stier and Sackmann, 1973; Klausner et al., 1980; Karnovsky et al., 1982; Simons and Sampaio, 2011). Also, confinement by microdomains would allow receptors and cofactors to meet faster and therefore speed up cell responses (Batada et al., 2004). Clustering of receptor tyrosine kinases (RTKs) upon ligand binding has been shown to be essential for the activation of kinases, which promote downstream signaling cascades (Saha et al., 2007). Ephrin receptors form the largest subfamily of RTKs regulating cell shape, movement and attachment. Upon binding to Ephrin ligands, Ephrin receptors accumulate into highly packed microdomains, which generate clearly defined signaling centers at cell–cell interfaces (Saha et al., 2007; Seiradake et al., 2010). Perturbation of Ephrin receptor clustering by point mutation in the binding interfaces of extracellular

domains results in homogenous cell surface distribution with a loss of clusters at cell–cell contacts and yields disruption of signaling cascades (Seiradake et al., 2010). Another important member of RTKs is the epidermal growth factor receptor (EGFR), which is implicated in cell growth, proliferation, and differentiation to cell survival (Ullrich and Schlessinger, 1990; Yarden and Sliwkowski, 2001; Hoeller et al., 2005). Binding of EGF to its receptor EGFR leads to receptor dimerization, followed by tyrosine phosphorylations of the receptor (Pawson, 2004) and assembly of the protein complexes which activate intracellular signaling (Blagoev et al., 2003; Jones et al., 2006). In EGFR clusters, the number of phosphorylated EGFRs become larger than the number of EGF ligands, as unliganded EGFRs are also phosphorylated, implying an amplification of EGF signaling (Ichinose et al., 2004). Microdomains also serve as platforms for receptor internalization, thus modulate the sensitivity of cell signaling or the affinity of cell–cell adhesion during tissue morphogenesis (Klaasse et al., 2008; Levayer et al., 2011). In addition, a micro-scale organization is hypothesized to be the entry port for viruses (Mañes et al., 2003) and plays important roles in immunological response (Dykstra et al., 2003). In calcium signaling, formation of Ryanodine receptor (RyR) clusters are required for Ca^{2+} sparks (Cannell et al., 1995), which is required for muscle contraction and neurotransmission (Baddeley et al., 2009). In the context of cell adhesion, microdomains, or clusters of adhesion molecules are essential for supporting tensile forces during cell migration (Maheshwari et al., 2000; Roca-Cusachs et al., 2009) and cell shape changes (Cavey et al., 2008; Rauzi et al., 2010). Together, these reports showed that the spatial organization of membrane proteins into microdomains can play crucial roles in a large range of biological processes.

Over the last few decades, a large number of studies on membrane models and extracted cell membranes have led to the hypothesis that cell membranes are heterogeneous and microstructured. The co-existence of liquid-ordered and liquid-disordered phases has been first documented on membrane models and extracted cell membranes by using different physical methods including electron spin resonance (ESR; Stier and Sackmann, 1973; Marsh et al., 1982; Ge et al., 2003; Swamy et al., 2006), different scanning calorimetry (DSC; Mabrey and Sturtevant, 1976; Melchior, 1986; Wolf et al., 1990), X-ray (Wunderlich et al., 1978; Gandhavadi et al., 2002), nuclear magnetic resonance (NMR; Mitrakos and MacDonald, 1996, 1997; Veatch et al., 2004) electron microscopy (Hui and Parsons, 1975; Hartmann et al., 1977; Prior, 2003). Biochemical methods such as detergent-soluble membranes and crosslinking assays (Brown and Rose, 1992; Cerneus et al., 1993) have been extensively used and often been over-interpreted as a criterion for the existence of lipid microdomains in cell membranes. *In situ* measurements using fluorescence microscopy methods, such as fluorescent polarization or fluorescent life time imaging microscopy (FLIM) used to study fluorescent lipid analogs have shown the co-existence of different lipid phases (Fiorini et al., 1988) and their organization in sub-resolution domains in the plasma membrane (Owen et al., 2012). In addition, the dynamics of membrane proteins revealed by fluorescence recovery after photobleaching (FRAP; Wolf et al., 1981; Metcalf et al., 1986), fluorescent correlation spectroscopy (FCS; Fahey et al., 1977; Meyer and Schindler, 1988; Korlach et al., 1999; Schwille et al., 1999), and single particle tracking (SPT) using optical (Kusumi et al., 1993) or fluorescent labels (Schutz et al., 2000; Jaqaman et al., 2011; Suzuki et al., 2012) have demonstrated multiple modes of diffusion: different diffusion coefficients or different types of motion (i.e., confined/Brownian) for a single protein species (Metcalf et al., 1986; Schwille et al., 1999) or for lipid analogs (e.g., saturated and unsaturated lipid probes) which partition in different lipid phases (Wolf et al., 1981; Schutz et al., 2000). These observations prime the hypothesis that there are local heterogeneities, such as "pinball in pinball machine" (Jacobson et al., 1995; Sheets, 1995) with microdomain obstacles or "membrane-skeleton fences" (Kusumi et al., 1993; Sako and Kusumi, 1994) mediated by protein-cytoskeleton interactions. Moreover, at the molecular scale (i.e., 2–10 nm), Förster resonance energy transfer (FRET) experiments have supported the existence of small tightly packed clusters of membrane anchored and transmembrane proteins with size of few 10s nanometers (e.g., <70 nm in case of GPI-anchored proteins) containing only few proteins (Damjanovich et al., 1997; Varma and Mayor, 1998; Sharma et al., 2004; Gowrishankar et al., 2012).

While current data strongly support the existence of microdomains/clusters of different kinds, some of the methods cited above are prone to artifacts or have various limitations when used to characterize microdomains. First, concerning the spectra-based methods (e.g., ESR, DSC, X-ray, NMR), the calibrated spectra obtained from very simple membrane models composed of only a few types of lipids at predefined ratio are often too simplistic to interpret the spectra obtained on cell membranes, which are far more complex in terms of lipid and protein compositions. Second, biochemical methods, such as nonionic detergent-soluble assays, can induce artificial clustering (Heerklotz, 2002). Third, although capable of providing nanometric resolution, electron microscopy suffers from low specificity and artifacts caused occasionally by long and invasive sample preparation. Fourth, the interpretation of FRAP and FCS data are generally model-dependent. Fifth, SPT cannot always distinguish between alternative models of membrane organization, if they show similar single molecule dynamics. Finally, while the conventional fluorescence imaging methods such as confocal microscopy provide direct imaging of membranes *in vivo*, they fail to resolve domains of nanometric sizes and cannot be used to assess the models inferred from FRET or FCS measurements. This failure arises from the fundamental limit of diffraction, which sets a criterion of the minimum resolvable distance between two punctual objects (Abbe, 1873; Rayleigh, 1874). Molecules closer than this limit, ∼200–350 nm (for optical wavelengths), cannot be distinguished. Thus, new methods that manage to circumvent this optical limit are required for direct visualization and quantification of nanoscopic organization of membrane domains. Fortunately, during the last few years, different strategies have been proposed and have successfully improved the spatial resolution to one tenth of the diffraction limit. In the following sections, we will review these so-called "superresolution" optical methods and discuss how they contribute to our understanding of the mesoscopic organization of the plasma membrane. While, little has been done yet with these new approaches on the membranes of plant cells, recent works on

animal cells that we present here will hopefully pave the way for the plant community.

SUPERRESOLUTION USING SPATIAL MODULATION

Improvement of the resolution can be obtained by spatial modulation of the excitation light. By exciting the sample plane with a series of patterns, structured illumination microscopy (SIM) can decode the conventional inaccessible high-resolution structural information into Moiré images obtained by individual excitation pattern (**Figures 1A,D**) and then allow the reconstruction of images at higher resolution (Gustafsson, 2000). This method allows to achieve a twofold increase in resolution in 2D (Gustafsson, 2000) or 3D (Gustafsson et al., 2008) with linear excitation, and even a theoretical unlimited increase in resolution in the nonlinear excitation regime (Gustafsson, 2005; Rego et al., 2012). SIM has been used to visualize the punctate organization of antigen membrane glycoprotein (Hammonds et al., 2012), lipid rafts (Svistounov et al., 2012) of 100 nm in size and structure of nanopores in plant cells (Fitzgibbon et al., 2010). Yet, there are two main challenges for SIM. First, to improve the resolution, multiple ($10 \sim 100$) excitation patterns are required per imaging plane, thereby fundamentally limiting the acquisition rate. Second, the reconstruction of superresolution image requires complicated and time consuming computational post-analysis, in particular for 3D image. Note that recent analog implementation using microarray lenses for on-line optical analysis can eliminate the need to acquire and digitally combine multiple camera exposures, thereby improving time-resolution down to few 10s milliseconds (York et al., 2013).

Resolution can also be increased in another elegant way. In fluorescence microscopy, the size of the focal spot at the sample plane usually defines an unresolvable region. The resolution can, however, be improved by reducing the size of the region from which the excited molecules fluoresce. By superimposing a hollow-patterned laser (e.g., donut-shape) on the conventional excitation laser to specifically quench excited molecules at the rim of the focal spot through stimulated emission (**Figures 1B,D**), stimulated emission depletion (STED) microscopy can improve the lateral resolution down to a few ten nanometers (Hell and Wichmann, 1994; Klar et al., 2000; Westphal and Hell, 2005). Furthermore, the same principles can be applied to improve the axial resolution with a depletion phase mask acting along the optical axis. Combination of lateral and axial depletion beams allows to obtain isotropic resolution (3D-STED, Harke et al., 2008; Hein et al., 2008; Wildanger et al., 2009). 2D-STED microscopy has revealed a large set of synaptic or membrane-bounded protein microdomains of 50–60 nm in size (Sieber et al., 2006, 2007; Willig et al., 2006; Demir et al., 2013). Furthermore, by implementing FCS in variable STED nanometric observation volumes, as done previously with diffraction-limited spots (Wawrezinieck et al., 2005; Lenne et al., 2006), STED microscopy has further characterized the size (<20 nm) and lifetime of lipid rafts (∼10 ms; Eggeling et al., 2009). While STED can practically increase resolution by five fold as compared to classical confocal microscopy, it is limited in the speed of acquisition (∼0.1–1 Hz). High laser power of the depletion laser (10^4–10^7W/cm^2 for pulse peak intensity) can be very toxic for live samples and can cause photo-bleaching during imaging. Finally, the efficiency of STED effect requires a perfect alignment of the excitation and the depletion lasers, which might be complex to achieve.

SUPERRESOLUTION USING TEMPORAL MODULATION

Spatial resolution can alternatively be improved by modulating/switching the emission of fluorescent molecules. The rationale of this approach is that the position of a single fluorescent molecule can be determined with a precision much better than the resolution criterion imposed by the diffraction limit, if the number of collected photons per molecule is high (Thompson et al., 2002). In a dense material, simultaneously excited molecules separated by distances smaller than the diffraction limit cannot be individually localized due to the spatial overlap of their fluorescence signal. If only a sparse subset of fluorescent molecules, separated by distances larger than the diffraction limit is activated at one time of acquisition, they can be localized individually with high precision. The whole population of fluorescent molecules can thus be localized by successive acquisitions, using temporal modulation/switching of fluorescence emission, thereby providing a map of single molecules and an image at super-resolution (**Figures 1C,D**). To date, there are two types of microscopies, which implement these principles using photoswitchable molecules. The first type called photoactivated-localization microscopy (PALM), is based on photoactivable fluorescent proteins (PAFPs; Betzig et al., 2006). Upon irradiation by appropriate activation laser (e.g., UV laser), PAFPs can shift their spectral emission from one color to another [e.g., EosFP (Wiedenmann et al., 2004), Dendra (Gurskaya et al., 2006)] or from dark to bright states [e.g., Dronpa (Ando et al., 2004), PamCherry (Subach et al., 2009)]. The second type called stochastic optical reconstruction microscopy (STORM; Rust et al., 2006), is based on photo-switchable organic probes: fluorescent activator/reporter probe (e.g., cy3/cy5) can undergo multiple fluorescent cycles between dark and bright states triggered by excitation and activation lasers (e.g., 657/532; Rust et al., 2006). In a variant form of STORM called "direct" STORM (dSTORM), conventional fluorophores can also be "directly" reversibly recycled between fluorescent and dark states by irradiation with a single wavelength and the use of a reducing buffer without any need of activator fluorophore (Heilemann et al., 2008). The axial resolution along the optical axis can also be greatly improved up to a few 10 nm using astigmatic detection (Huang et al., 2008), bi-plane (Juette et al., 2008), or more sophisticated interfometric methods (Shtengel et al., 2009; Kanchanawong et al., 2010). In live mode for slowly moving structures, PALM/STORM has provided kinetic data on nanoclusters with a spatial resolution of 60 nm and a time resolution down to 25 s (Shroff et al., 2008). Alternatively, by tracking photoactivated molecules, single particle tracking-PALM (sptPALM) can obtain few orders of magnitude more trajectories per cell in comparison with traditional SPT, therefore can create a map of the dynamic heterogeneity in cell membrane (Manley et al., 2008). Furthermore, if molecules are photoactivable only once, PALM provides quantitative counting of single molecules, thereby allowing the measurement of the density or even the stoichiometry of microdomains' components.

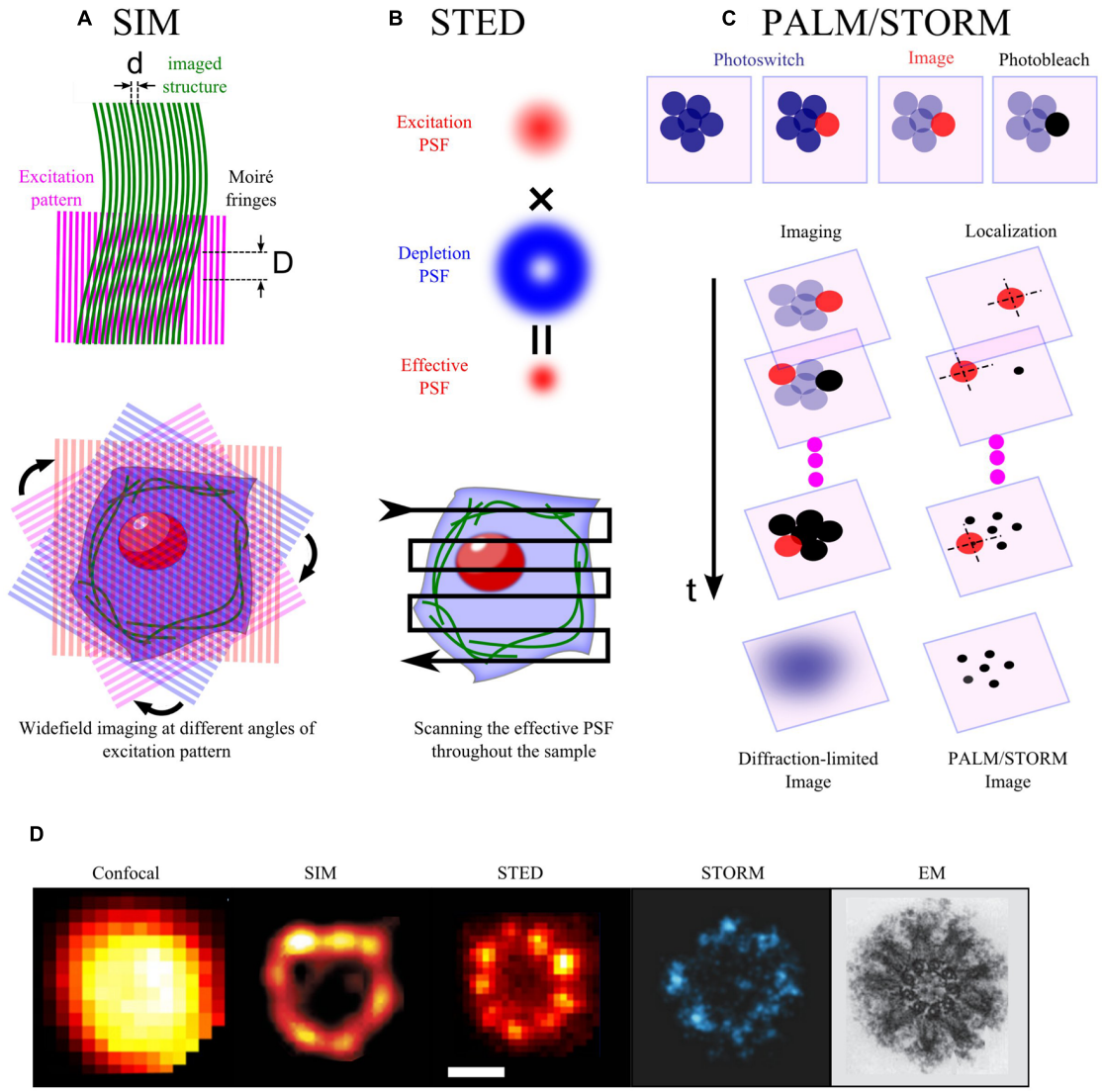

FIGURE 1 | Basic principles of some superresolution optical methods. (A) When two line patterns are superposed multiplicatively, a beat pattern or Moiré fringes are formed with smaller spatial frequency than the initial patterns (upper panel, $d < D$). In SIM, the sample plane is excited by a patterned wide-field illumination (commonly in striped-shaped pattern). This pattern combines with the sample structural pattern to form Moiré fringes. Thus, high spatial frequency information of the sample, which is below the diffraction limit, is decoded into a lower frequency Moiré pattern. Moiré patterns obtained by rotating the excitation pattern at different angles are collected and used for a computational reconstruction of a higher resolution images (lower panel). **(B)** In STED, the sample plane is illuminated simultaneously by an excitation and a depletion beam. The depletion beam in a donut-shape allows to deplete non-linearly most of the excited molecules into the dark state through stimulated emission, but leaves intact the center of the excitation PSF. This thus yields an effective PSF, which is smaller than the initial diffraction-limited PSF. The sample is scanned with this effective PSF to form a superresolution image. **(C)** Single molecules in a crowded biological sample can be isolated by photoswitching (from dark to bright or from one color into another). The photoswitched molecules are then imaged and localized before being photobleached (upper panel). The procedure is iteratively repeated until all of the initial molecules are imaged and photobleached. The superresolution image is reconstructed from the position list of all localized molecules (lower panel). **(D)** Images of the centriole taken with different methods: confocal and STED images (Lau et al., 2012), SIM image (Lawo et al., 2012), STORM image (Mennella et al., 2012), EM image (Hagan and Palazzo, 2006).

Although so far, localization-based microscopy (PALM/STORM) has provided the best spatial resolution among other super-resolution optical microscopy techniques, its time resolution is still limited (>0.05 Hz) due to the need to collect a large amount of single molecule images. Furthermore, PALM/STORM data require cautious interpretation. There are indeed concerns about clustering artifacts, which might arise from the oligomeric nature of fluorescent tags (McKinney et al., 2009; Zhang et al., 2012). Moreover, some fixation methods fail to immobilize lipids and to a less extent lipid anchored proteins and some signaling proteins (Tanaka et al., 2010). Clustering could then arise from artificial antibody-induced processes. In addition, fluorescent molecules can exhibit photoblinking, which results in artificial clustering due to multiple detection of the same molecule (Annibale et al.,

2011). Finally, localization of single molecules in a high background noise, correction for sample drift during long acquisition periods (10–30 min) and data visualization/analysis from the list of localized proteins are often complicated and time consuming.

The development of localization microscopy (PALM and STORM) is currently a very active field with rapid development in different areas. The possibility to photoswitch a large range of commercially available fluorescent tags using reducing buffer without the need of oxygen scavengers (Heilemann et al., 2009; van de Linde et al., 2011) makes it an extremely convenient method for multicolor superresolution imaging. In addition, coupling of PALM and dSTORM provides simultaneous access to molecular counting of PALM and higher resolution imaging of dSTORM which use brighter and less photobleaching organic fluorophores compared to fluorescent proteins (Izeddin et al., 2011; Muranyi et al., 2013). Brighter versions of fluorescent proteins [mEos3 (Zhang et al., 2012), PSmOrange2 (Subach et al., 2012), mGeos-M (Chang et al., 2012)] and fluorophores (Lukinavičius et al., 2013), optimization of imaging buffer [e.g., Heavy water (Lee et al., 2013), cyclooctatetraene (Olivier et al., 2013a), Vectashield (Olivier et al., 2013b)], and labeling strategy [e.g., Nanobody (Ries et al., 2012)], have pushed further the spatial and temporal resolution limits.

In parallel with the improvement of imaging techniques, new algorithms for image analysis have been proposed. Real-time analysis requires the implementation of new algorithms for fast detection and localization of single molecules from large series of images. Localization is speed-up by replacing the classical Gaussian kernel-based fitting algorithm by the classical Högbom "CLEAN" algorithm in QuickPALM (Henriques et al., 2010), the fluoroBancroft algorithm in livePALM (Hedde et al., 2009), radial symmetry center (Parthasarathy, 2012), or wavelet segmentation (Kechkar et al., 2013) and also by implementation of parallel computational structures such as graphical processing unit (Smith et al., 2010). In addition, the precision of single molecule localization in a highly dense sample, in particular for STORM, has been significantly increased. Single molecule positions are retrieved by fitting overlapped spots with a multiple PSF, either using a maximum likelihood estimation in DAOSTORM (Holden et al., 2011), Bayesian statistics (Cox et al., 2012) or a global optimization using compressed sensing, which does not require any assumption on the number of molecules in the image (Zhu et al., 2012). Alternatively, the superresolution image can be obtained by iterative image deconvolution in place of single or multiple emitter localization (Mukamel et al., 2012). Furthermore, new toolboxes for analyzing complex patterns of protein organization using pair-correlation analysis (e.g., PC-PALM, Sengupta et al., 2011, 2013; Veatch et al., 2012) or for visualization of 3D PALM/STORM data using surface rendering (Beheiry and Dahan, 2013) are now available to the scientific community. The use of monomeric fluorescent tags (Zhang et al., 2012), monovalent antibodies or purified Fab fragments (Chojnacki et al., 2012), and new computational algorithm for photoblinking correction (Annibale et al., 2011; Lee et al., 2013) have significantly reduced clustering artifacts. In the near future, the combination of PALM/STORM with EM (Watanabe et al., 2011) or FRET (Renz et al., 2012) will be very useful to characterize the supramolecular organization membrane microdomains.

NANOSCOPIC ORGANIZATION: A SHARED FEATURE BY LIPIDS AND PROTEINS

During the past decades, the study of membrane organization has been mainly focused on lipid organization, the putative lipid rafts being emphasized as a "stereotype" of membrane domains. Focus on lipid rafts has masked to some extent the existence and the role of protein clustering in membranes. In addition, the lack of direct visualization evidence together with the recognition of possible experimental artifacts has raised doubts about the existence of microdomains/clusters.

Importantly, superresolution optical microscopy has supported the raft-hypothesis by providing direct evidence of lipid rafts *in vivo* as well as characterization of their dynamics when used in combination with other F-approaches such as FCS or FLIM (Eggeling et al., 2009; Mizuno et al., 2011; Owen et al., 2012). Superresolution imaging has also provided evidence of the nanoscopic organization of a large set of membrane proteins, ranging from immune (Lillemeier et al., 2010; Sherman et al., 2011; Scarselli et al., 2012), adhesion (Betzig et al., 2006; Shroff et al., 2007), viral (Manley et al., 2008; Lehmann et al., 2011), synaptic (Willig et al., 2006; Sieber et al., 2007), and chemotaxis (Greenfield et al., 2009) protein clusters (**Figures 2A–D**; **Table 1**). These microdomains/clusters have been observed not only in fixed but also in live cells (**Figure 2E**; Hess et al., 2007; Shroff et al., 2008; Hein et al., 2010) ruling out the possibility of artifacts caused by the fixation procedure (Hess et al., 2005; Lee et al., 2011). Interestingly, some proteins, such as adrenergic receptor β2-AR or viral protein HIV-1, do not colocalize with lipid raft markers suggesting raft-independent clustering (Lehmann et al., 2011; Scarselli et al., 2012). Thus, further application of multicolor superresolution and specific perturbation of lipid/protein to explore the contribution of protein–protein versus protein–lipid interactions to the formation of protein microdomains would be extremely informative.

THE DYNAMICS OF MICRODOMAINS

The possibility to observe nanoscopic domains in live cells has brought unprecedented information on their dynamics. Live-superresolution microscopy has been used to demonstrate a wide range of morphological and dynamic behaviors, which depend on the types of proteins, subcellular environments, and cell types. For example, adhesion complexes (e.g., Paxillin) have been shown to form either elongated structures with size up to few micrometers or point-like puncta of 100–300 nm in size. While elongated nascent adhesion complexes exhibit growth, fusion and dissolution at few minutes time scale, the punctae can be stable during a few 10s of minutes. The dynamics of theses structures differs from one cell type to another (e.g., CHO and 3T3 fibroblast cells) with different protrusive motions (Shroff et al., 2008). Some other types of membrane microdomains have much higher dynamics. The assembly of endocytic cargo proteins (e.g., Transferrin) is on the time scale of a few 10s of seconds, and the life time is on the time scale of 1 min (Jones et al., 2011). Furthermore, single molecule tracking has revealed heterogeneities in membrane protein dynamics. While non-clustering proteins (e.g., VSVG-protein) exhibits a rather homogenous diffusion map, clustering proteins (e.g., Viral protein Gag) show distinct zones of free diffusion and

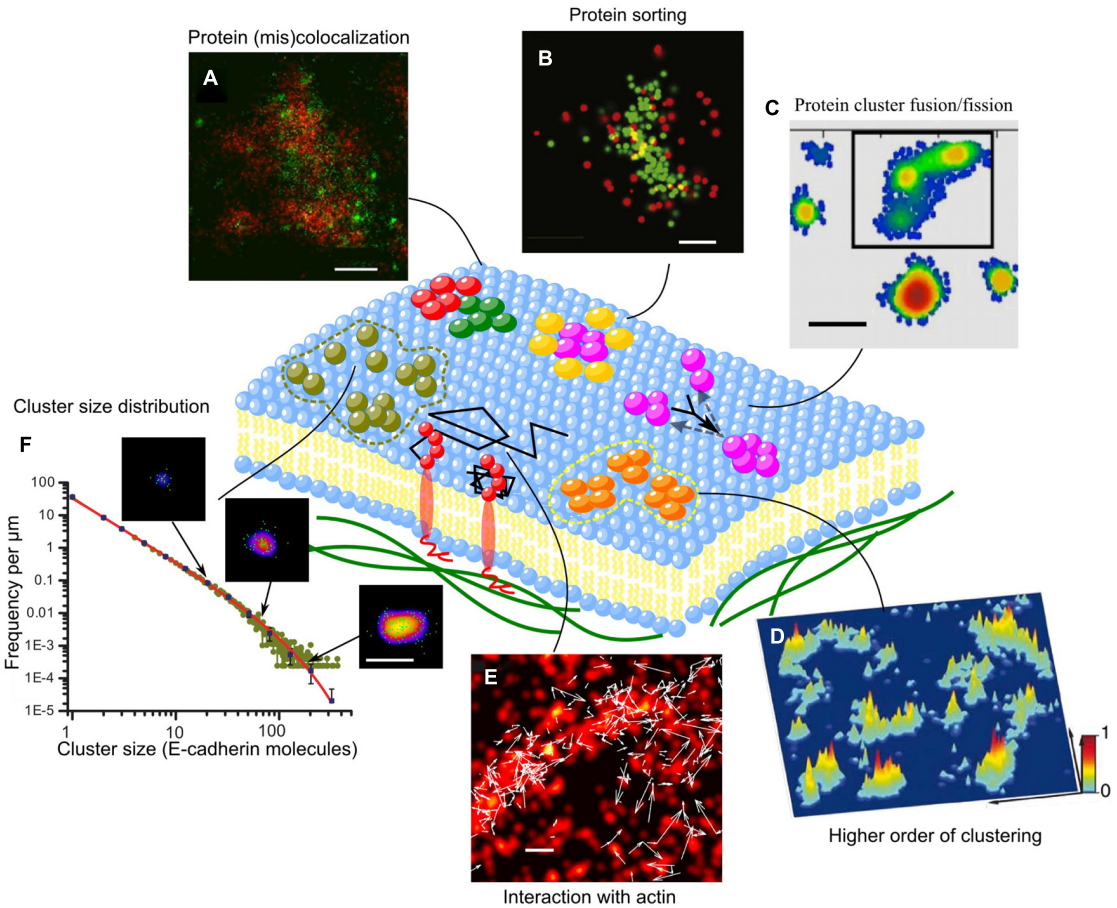

FIGURE 2 | Supramolecular organization and dynamics of membrane proteins. (A) α-actinin and vinculin only partially colocalize within each focal adhesion. While α-actinin exists in large patches emanating from stress fibers, vinculin coalesces in small, dense clusters scattered across each focal adhesion (Shroff et al., 2007). **(B)** Images of individual LAT clusters showing preferential organization of SLP-76 at the rims of LAT clusters (Sherman et al., 2011); **(C)** Plot of a representative membrane showing the distribution and inner gradients of several syntaxin clusters. Black frame: a super-cluster composed of three smaller clusters that might be in the process of uniting (Bar-On et al., 2012); **(D)** Three-dimensional probability density plots for CD3ζ-PSCFP2 in native plasma membrane sheets from activated T cells on immobilized surfaces. Molecules are presented as a normalized Gaussian probability density distribution with a width equal to their positional accuracy. Height and color represent the probability density at that point (*x*, *y*), with the highest probability density of all images set as 1 (Lillemeier et al., 2010); **(E)** Superimposed two-color (Dendra2-HA and PAmCherry-actin) live cell FPALM image of PAmCherry-actin (red pseudocolor) and trajectories of Dendra2-HA molecules (white arrows) in a living NIH3T3-HAb2 fibroblast at 37°C (Gudheti et al., 2013); **(F)** Cluster size distribution of E-cadherin clusters and the power-law with exponential cut-off fitting (solid line). Sample images of clusters are shown with arrows that indicate cluster size (Truong-Quang et al., 2013). Scale bars, 500 nm **(A)**, 200 nm **(B,C,E,F)**, and 1 μm **(D)**.

immobile behavior, suggesting a protein-trapping mechanism by microdomains (Manley et al., 2008). In this way, microdomains of vesicular decorating proteins, such as Clathrin, can recruit specific cargo proteins (e.g., Transferrin receptor TfR and epidermal growth factor receptor EGFR) with specific targeting sequence (Subach et al., 2010). Protein-trapping can be mediated by passive protein–protein interactions inside microdomains, but can also arise from interactions with the underlying active actin cytoskeleton (**Figure 2E**; Gudheti et al., 2013).

NANOSCOPIC ORGANIZATION OF MULTI-COMPONENT MICRODOMAINS

Superresolution optical microscopy has made possible the simultaneous visualization of different protein organization at the nanoscopic scale (**Table 1**). Measuring the relative distance between protein components in complexes is key to determining the existence of molecular interactions. Some proteins, which colocalize at the diffraction-limited resolution turn out to have little overlap or even to create interwoven arrangements as revealed by high-resolution image (Shroff et al., 2007). Proteins that have similar functions (e.g., vinculin and alpha-actinin) show some degree of nanostructural overlap, while functionally distinct proteins (e.g., Paxilling and actin) exhibit very little overlap (**Figure 2A**). Also, proteins that were suggested to be in separate microdomains as observed by immunoelectron imaging, a method which is prone to clustering artifact (D'Amico and Skarmoutsou, 2008), turn out to have a significant overlap depending on the activating cell state. As proteins interact at short distances, spatial overlap at the nanometer scale is a condition to direct biochemical reactions (Lillemeier et al., 2010; Sherman et al., 2011),

Table 1 | Applications of superresolution microscopy to detection and quantification of the supramolecular organization in the plasma membrane.

Measurements	Methods	Spatial resolution	Temporal resolution	Observations and reference
Dimensions	SIM	100–150 nm in *x–y*, 200–300 nm in *z*	na	70–150 nm clusters of the antiretroviral membrane protein tetherin (Hammonds et al., 2012); 100 nm lipid raft microdomains (Svistounov et al., 2012). 200–500 nm clusters of membrane-bounded proteins in transgenic tobacco cells (Fitzgibbon et al., 2010).
	STED	50–70 nm	na	70–80 nm clusters of the calcium sensor synaptotagmin (Willig et al., 2006); 50–60 nm clusters of the vesicle docking protein Syntaxin (Sieber et al., 2006, 2007); 150 nm clusters of the cAMP signaling protein ACIII (Yang et al., 2013); 100 nm nanoclusters of the phos-phatase protein ABI1 and protein kinase CPK21 in *Arabidopsis thaliana* cells (Demir et al., 2013).
	PALM	10–30 nm	na	100–150 nm clusters of Vinculin in focal adhesions (Betzig et al., 2006); 100 nm lipid raft (Mizuno et al., 2011); 100 nm clusters of the G-protein receptor GPCR (Scarselli et al., 2012, 2013); 100 nm clusters of the retroviral protein Gag and antiretroviral protein tetherin (Lehmann et al., 2011); 35–70 nm clusters of the T cell receptor (Lillemeier et al., 2010).
	STORM	20–30 nm	na	65–105 nm clusters of viral envelope protein Env (Roy et al., 2013); <300 nm clusters of immune receptor TLR4 (Aaron et al., 2012).
Multicomponent organization	STED	40 nm	na	Different clustering states of the viral envelope protein Env around the core protein Gag, corresponding to different maturation stages of viral particles (Chojnacki et al., 2012); Synapsin forms cluster inside or outside synaptic vesicles (Kempf et al., 2013).
	PALM	20–30 nm	na	Different levels of colocalization of the cargo protein transferin with the vesicle coat clathrin (Subach et al., 2009); nanoclusters of vinculin, paxillin, zyxin (in focal adhesion) have interwoven arrangements with little overlap (Shroff et al., 2007); the adaptor protein SLP-76 localizes at the periphery of immune protein Lat nanoclusters (Sherman et al., 2011).
	PALM/STORM	15–20 nm	na	The highly adhesive isoform of AQP-4 (aquaporin channel) forms the core of 50–130 nm clusters and is surrounded by a less adhesive isoform (Rossi et al., 2012); 50–150 nm HIV-Gag clusters are surrounded by the viral envelope protein Env (Muranyi et al., 2013).
	iPALM	<20 nm isotropic	na	Focal adhesion complexes have a three-layer structure: a membrane-apposed signaling layer containing integrin, focal adhesion kinase, and paxillin; an intermediate force-transduction layer containing talin and vinculin; and an uppermost actin-regulatory layer containing zyxin, vasodilator-stimulated phosphoprotein and alpha-actinin (Kanchanawong et al., 2010).
Multiscale organization	STED	40 nm	na	40–60 nm adhesion clusters spaced by 100 nm, accumulate inside focal adhesions of few micrometers (Rönnlund et al., 2013).
	PALM	20–30 nm	na	100–200 nm clusters of vinculin, paxillin, zyxin accumulate to form focal adhesions of few micrometers (Betzig et al., 2006; Shroff et al., 2007); the intercellular adhesion protein E-cadherin forms clusters of a few 10s to a few 100s molecules, which accumulate into micrometer adhesion puncta (Truong-Quang et al., 2013). 100 nm clusters of the immune protein TCR, LatA, ZAP-70 in immunological synapses of a few micrometers

(Continued)

Table 1 | Continued

Measurements	Methods	Spatial resolution	Temporal resolution	Observations and reference
				(Lillemeier et al., 2010; Sherman et al., 2011); nanoscopic clusters of Tar, CheY, CheW accumulate at the two ends of *E. coli* bacteria to form micrometer-scale clusters (Greenfield et al., 2009); The viral protein hemagglutinin forms clusters with size ranging from 40 nm up to a few micrometers (Hess et al., 2007).
	dSTORM	20 nm	na	Syntaxin 1 or SNAP-25 (synaptic proteins) form 90–130 nm clusters, whose molecular density gradually decreases from the core to the periphery. Large-clusters show several density gradients, suggesting that they are formed by fusion of several clusters (Bar-On et al., 2012).
Kinetics of assembly/disassembly	SIM	100 nm	s	Dynamic assembly of the membrane bound DNA translocase SpollIE protein, in *B. subtilis* bacteria (Fiche et al., 2013).
	Live STED	40 nm	10 s	Clusters of the cell membrane proteins caveolin and connexin-43, from 50 to a few hundreds nanometers in size in living cells (Hein et al., 2010).
	FCS-STED	30 nm	<us	Anomalous diffusion of lipid analogs in membrane models or in living cells (Eggeling et al., 2009; Mueller et al., 2011; Leutenegger et al., 2012; Sezgin et al., 2012; Honigmann et al., 2013).
	Live PALM	60 nm	25 s	100–300 nm clusters of the focal adhesion protein Paxillin exhibit growth, fusion, and dissolution on the order of a few minutes to a few 10s of minutes time scale (Shroff et al., 2008).
	sptPALM	20–30 nm	30–100 ms	Dynamic heterogeneity of the viral protein Gag with a mobile fraction and an immobile fraction confined in 100–200 nm clusters (Manley et al., 2008); the trajectories of the cargo proteins TfR or EGFR overlap those of the vesicle coat Clathrin (Subach et al., 2010); Colocalization of the (non-mobile) Hemagglutinin viral protein with actin-rich membrane regions (Gudheti et al., 2013).
	Live STOM	30 nm in x–y; 50 nm in z	0.5–2 s	Dynamic assembly of endocytic vesicles with 70 nm clusters of the cargo protein Transferrin, surrounded by 150 nm clusters of the vesicle coat Clathrin (Jones et al., 2011).
Cluster size (number of molecules) distribution	PALM	20–30 nm	na	Size distribution ranging from a few to a few 10s of proteins of the antiretroviral protein Tetherin (Lehmann et al., 2011); the T cell receptor (Sherman et al., 2011; Lillemeier et al., 2010; a G-protein signaling receptor (Scarselli et al., 2012); exponential distribution of chemotaxis proteins Tar, CheY, CheW (Greenfield et al., 2009); power-law distribution of the cell–cell epithelial adhesion protein E-cadherin (Truong-Quang et al., 2013).
	STED	50 nm	na	Clusters of ∼75 syntaxin molecules (Sieber et al., 2007); clusters of ∼7–10 of viral envelope protein Env trimers (Chojnacki et al., 2012).

which might then initiate massive recruitment, e.g., by docking of synaptic vesicles at the reaction site, and activation of downstream signals (Purbhoo et al., 2010; Williamson et al., 2011). Different levels of colocalization between nanoclusters of proteins, such as vesicular coats and cargo proteins, are indicative of different stages of maturation of the endocytic machinery (Subach et al., 2009). Moroever, multi-protein microdomains can have internal structure: for example the Adaptor protein SLP-76 localizes at the rim of nanoclusters of the immune protein LAT (**Figure 2B**; Sherman et al., 2011), suggesting a protein-sorting mechanism. The peripheral proteins prevent further accumulation of the core proteins and therefore control the growth of microdomains, as being reported for nanoclusters of aquaporin channel (Rossi et al., 2012). Furthermore, protein complexes in a microdomain can be

structured along the transverse direction into nanoscopic composite multilaminar protein architecture. For example, focal adhesion complexes are composed by three layers: a membrane-apposed signaling layer containing integrin, focal adhesion kinase and paxillin; an intermediate force-transduction layer with talin and vinculin; and an uppermost actin-regulatory layer with zyxin, vasodilator-stimulated phosphoprotein, and alpha-actinin (Kanchanawong et al., 2010).

MULTISCALE ORGANIZATION OF MICRODOMAINS

Protein–protein interactions and cooperation depend critically on their relative distance, and therefore on molecular packing in microdomains. In fact, changes in lateral packing of chemoreceptor arrays significantly affect bacterial chemotaxis response (Khursigara et al., 2011). Similarly, modulation of intermolecular distances between the cell-matrix adhesion protein integrin using nanopatterned substrates can amplify or suppress the adhesion force (Selhuber-Unkel et al., 2010). Although, the resolution limit of PALM/STORM (e.g., 20 nm) is larger than the size of most membrane proteins (e.g., ∼5–7 nm), molecular packing/density in clusters can still be inferred from the the number of proteins counted in a cluster area. As an example, based on quantitative PALM data, the clusters of the cell–cell adhesion molecule E-cadherin was found to be tightly packed *in vivo* (Truong-Quang et al., 2013). Molecular packing of microdomains can vary dependent on the type of proteins (e.g., Between GPI-anchored protein and signaling protein Lyn, Lat; Sengupta et al., 2011). Density of molecules can be also significantly different in the center and at the periphery of microdomains (Bar-On et al., 2012).

Interestingly, for a large range of proteins, highly packed microdomains do not distribute randomly but tend to form larger clusters, that may explain previous observations of larger microdomains at the microscale [e.g., Immune synapse (Monks et al., 1998; Davis et al., 1999) or adhesion complexes (Zaidel-Bar et al., 2003; McGill et al., 2009)]. For example, nanoclusters of cell-matrix adhesion complexes (e.g., Vinculin, Paxillin, Zyxin) of 100–200 nm in size (Betzig et al., 2006; Shroff et al., 2007) accumulate to form focal adhesion of few micrometers in size. Similarly, immune proteins (e.g., TCR or LatA) form clusters of a few 10s of nanometers, which concentrate, with a proximity of about 100 nm, in the micrometer-scale immune synapse (**Figure 2D**; Lillemeier et al., 2010). The same observation has been made for the viral protein Hemagglutinin (Hess et al., 2007) and chemoreceptors (Greenfield et al., 2009). Higher order clustering can facilitate the growth of microdomains by fusion. Indeed, for the synaptic protein Syntaxin, while the small clusters exhibit a gradual decrease in density from the core to the periphery, larger clusters show several density gradients suggesting that these large-clusters are formed by the fusion of several smaller clusters (**Figure 2C**; Bar-On et al., 2012). Tuning the relative distance between microdomains is likely to provide an effective way to modulate biochemical reactions (Lillemeier et al., 2010; Sherman et al., 2011).

VARIATION IN CLUSTER SIZE DISTRIBUTION

One of the major goals of studies on microdomains is to understand the mechanisms underlying domain formation and regulation. Among the physical observables, cluster size is a key to delineate the different theoretical models, which predict its distribution and how this varies upon changes of parameters such as protein concentration, recycling, or binding rate (Israelachvili, 1985; Turner et al., 2005; Meilhac and Destainville, 2011). Measuring distribution of cluster size is a challenge that PALM microscopy can meet, thanks to molecular counting. Interestingly PALM microscopy has revealed that microdomains can show various types of cluster size distribution. While some membrane proteins (e.g., antiretroviral protein Tetherin, immune protein TCR, G-protein signaling receptor) form cluster with characteristic size of a few to a few 10s of molecules (Lillemeier et al., 2010; Lehmann et al., 2011; Scarselli et al., 2012), others (e.g., Chemotaxis receptor and intercellular adhesion protein) form clusters which follows exponential (Greenfield et al., 2009), or power-law distribution (**Figure 2F**; Truong-Quang et al., 2013). Different types of cluster size distributions reflect distinct mechanisms of formation. An exponential distribution of cluster size, suggests stochastic self-assembly by random receptor diffusion and receptor–receptor interaction (Greenfield et al., 2009), while a power law distribution with exponential cut-off indicates that the size of clusters is regulated not only by dynamical fusion and fission processes but also by endocytosis (Truong-Quang et al., 2013). Analysis of cluster size distributions requires stringent handling as measured distributions can be biased by image processing. For instance, thresholding (consider or eliminate clusters smaller than a certain size) can lead to contradictory conclusions on cluster size (Sherman et al., 2011).

CONCLUDING REMARKS

Emerging optical methods have provided a powerful palette to study different aspects of membrane microdomains (**Table 1**). Superresolution optical methods form currently an active field with fast and continuous improvements. In a near future, it is likely that we will gain access to the dynamics of objects with molecular scale precision (2–10 nm) at subsecond time scale. Moreover, combining super-resolution fluorescence microscopy for specificity and localization precision with EM or AFM for resolution will provide molecular details on the organization of supramolecular structures (Watanabe et al., 2011; Chacko et al., 2013). Combination with F-techniques such as FRET, FLIM, and FCS will be also critical to probe the conformation and dynamics of membrane components in microdomains. Thus, questions concerning various aspects of microdomains could be tackled. With a better resolution and multicolor visualization, one could directly visualize the arrangements of molecules within the microdomains. It will be interesting to examine whether molecules are regularly distributed in an array or randomly packed with hollow structure. Quantitative analysis, such as molecular counting, can be very useful to understand the mechanisms underlying domain formation in cell membranes. How the cluster size distribution changes with protein concentration or under perturbations of domain assembly/disassembly rates (e.g., by disruption or enhancement of the actin polymerization) and the endocytosis will help to falsify various theoretical models. Also, by exploring the possibility of the coupling of microdomains between the inner and outer leaflets of the plasma membrane, one could shed light on how information is transmitted through the bilayer. Can clustering of receptors (e.g.,

GPI-anchored proteins) in the outer leaflet trigger the rearrangement of downstream proteins (e.g., Kinase, phosphatase proteins) in the inner leaflet, thereby amplifying the signals? A similar question could be addressed in the context of cell–cell adhesion for two opposed membranes where cis- and trans-clusters form: are the processes of cis- and trans-clustering occur at the same time? Do cis-clusters pre-exist? Simultaneous visualization of protein clusters at two opposed cell membranes would help solving the above questions. Understanding clustering kinetics is also essential for the understanding on the growth and maintenance of microdomains. Higher temporal resolution will help to probe the dynamics, assembly and disassembly of microdomains in the cell membrane. How passive and active processes driven by trafficking and cytoskeletal interactions integrate to shape clusters is a challenging question, which is now within reach of superresolution methods.

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Protein diffusion in plant cell plasma membranes: the cell-wall corral

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Studying protein diffusion informs us about how proteins interact with their environment. Work on protein diffusion over the last several decades has illustrated the complex nature of biological lipid bilayers. The plasma membrane contains an array of membrane-spanning proteins or proteins with peripheral membrane associations. Maintenance of plasma membrane microstructure can be via physical features that provide intrinsic ordering such as lipid microdomains, or from membrane-associated structures such as the cytoskeleton. Recent evidence indicates, that in the case of plant cells, the cell wall seems to be a major player in maintaining plasma membrane microstructure. This interconnection / interaction between cell-wall and plasma membrane proteins most likely plays an important role in signal transduction, cell growth, and cell physiological responses to the environment.

Keywords: lateral mobility, plasma membrane microdomains, diffusion, corral, cell wall, cytoskeleton

INTRODUCTION

Due to thermal agitation, molecules diffuse within liquid environments. In the specific case of proteins anchored within membranes, their diffusion is restricted to the two dimensions of the membrane plane. This type of motion is called lateral mobility. The propensity of a protein to move within the plane of a membrane has important biological consequences in terms of membrane micro-organization, protein-protein interactions and signal transduction mechanisms. An example of this is receptor clustering within the plasma membrane (PM) of post-synaptic neurons, where lateral mobility is linked to nerve plasticity (for review see: Choquet and Triller, 2003). In plant biology, thanks to recent microscopical advances, links between spatial segregation of proteins and signal transduction have been made. For instance, abscisic acid signaling via inhibition of ABI1 (abscissic-acid instensitive 1) modulates, within micro-domains, the recruitment of a complex between the anion transporter SLAH3 (slow anion channel 1 homolog 3) and its regulatory calcium dependent protein kinase (CPK21) (Demir et al., 2013). Similarly, a recent study has shown that, upon ammonium treatment, ammonium transporter 1.3 (AMT1.3) forms clusters within the PM prior to activation of an endocytotic mechanism. This suggests that AMT1.3 dynamics play a functional role in the cell's response to NH^{4+} (Wang et al., 2013). These results illustrate the central role of protein lateral mobility in plant cell responses to their environment.

FRAP AND OTHER APPROACHES

One of the most popular approaches for study of protein lateral mobility is Fluorescence Recovery After Photobleaching (FRAP). FRAP is simple to do and yields results quickly when working with living cells. Membrane proteins tagged with fluorescent proteins are especially amenable to this technique. Fluorescence is bleached by a laser beam within a Region of Interest (ROI). Reappearance of fluorescence in the bleached ROI is then monitored over time. Mean fluorescence intensity within the ROI increases if fluorescent molecules are free to diffuse into it from the non-bleached surrounding region during the post-bleaching phase. This increase in mean fluorescence intensity can be fit with curve equations to yield information of the type of diffusion under study (Axelrod, 1983; Feder et al., 1996; Sprague et al., 2004). For example, one could ask, is the protein under study free to diffuse or is it constrained in its lateral mobility by interaction with another cellular structure? Two important quantitative values arise from the equation of a recovery curve fit to a set of FRAP data, and these describe the amount and rate of protein lateral mobility under study. The first of these, the "mobile fraction," describes the fraction of a given protein that is unconstrained and therefore free to diffuse within the membrane. Secondly, the "half time" ($t_{1/2}$) describes the rate at which diffusing molecules do so. Depending of the shape of the ROI, $t_{1/2}$ can be used to extrapolate a relative diffusion coefficient (Yang et al., 2010; Mai et al., 2013). Calculated

"half times," are useful for comparing mobilities of different proteins.

Interestingly, the fluorescence recovery curve of PM proteins describes the sum of at least two additive mechanisms: the lateral mobility of the protein within the PM, and the exchange of proteins between cytoplasmic vesicles and the PM by endocytosis and exocytosis. It is possible to distinguish between protein exchange on and off the membrane, and lateral diffusion by analysing fluorescence recovery images with the help of 1D Gaussian fits. To do this, fluorescence intensity is measured along a line fit through the center of the bleached region and plotted against distance from the center of the bleached spot. In the case of pure lateral diffusion in which fluorescence recovery is only possible from the margins of the bleached region, the Gaussian profile becomes more shallow and widens over time so that the area under the curve is conserved. Conversely, for the case of fluorescence recovery that is caused by protein addition to the membrane by exocytosis, the Gaussian curve maintains its width over time while its area is reduced. This is because exocytosis happens uniformly over the entire bleached region (Oancea et al., 1998; Hammond et al., 2009; Luu et al., 2012).

Recent advances in microscope technology and new fluorescent protein development now allow imaging of single fluorescent molecules in living membranes and consequently their dynamics are directly visible. These approaches have been successfully used on plant samples allowing recording of diffusion coefficients of PIP proteins (Li et al., 2011), and of the PM marker paGFP-LTI6b (Martinière et al., 2012). Super resolution microscopes which are capable of imaging at sub-diffration-limited dimensions have recently been used to image PIN auxin carrier recycling at the PM (Kleine-Vehn et al., 2011) and will change our vision of plant protein dynamics as has already happened in the neurosciences (Maglione and Sigrist, 2013).

PROTEIN LATERAL MOBILITY IN THE PLANT PLASMA MEMBRANE

Our knowledge about the lateral mobility of plant PM proteins is increasing very quickly. One of the first reports was on dynamics of the potassium channel KAT1 (Sutter et al., 2006). In that study, a fusion between KAT1 and photoactivable-GFP was used to record the lateral mobility of this protein. Measurement of protein dynamics using photoactivatable fluorescent proteins is like FRAP flipped on its head. The ROI starts out at 100% brightness after photoactivation and diffusion results in a decrease in its mean fluorescence intensity over time. In the case of KAT1, ROI fluorescence did not appreciably decrease, even after several minutes, suggestive of a very small mobile fraction; in other words, the potassium channel seems to be immobile within the PM. This very counterintuitive result was later confirmed for other PM proteins. Men et al. (2008) demonstrated that after 20 min PIN2-GFP only recovers to 40% of its pre-bleach intensity within an ROI. Similarly, FRAP experiments on BOR1, KNOLLE, NIP5.1, and CASP1 suggest very low lateral mobility of these proteins (**Table 1**) (Boutté et al., 2010; Takano et al., 2010; Roppolo et al., 2011).

As explained earlier, lateral diffusion of a protein is a direct consequence of temperature and its diffusion constant is dependent mainly on a protein's hydrodynamic radius and the viscosity of the membrane (Saffman and Delbrück, 1975). Numerous examples show high mobile fractions and diffusion coefficients from 0.1–1 $\mu m^2/s$ in animal cells (for review Owen et al., 2009). Plant cells are proving to be somewhat variable. For instance, GFP-AQP1 expressed in LLC-PK1 cells recovers to 100% pre-bleach brightness after 2 min (Umenishi et al., 2000) while GFP-PIP2.1, a plant homologue of AQP1, recovers to only 10% of its pre-bleach fluorescence after the same time (Luu et al., 2012). Many proteins in the plant PM seem to be constrained in their diffusion by mechanisms not found in animal cells. Sorieul et al. (2011) have compared the recovery curve of two AtPIP2;1-GFP constructs, one in the PM and the other carrying point mutations which cause it to be retained within the endoplasmic reticulum. Very low lateral mobility was only observed when the aquaporin is in the PM. This does not result from higher viscosity of plant PMs relative to other endomembranes like the ER because some proteins like LTI6b-GFP have high mobile fractions in the same conditions (**Table 1**) (Kleine-Vehn et al., 2011; Luu et al., 2012; Martinière et al., 2012).

A CELL WALL CORRAL

A central question in our research, then, is how and why are some PM proteins fixed in place while physical laws would dictate that they move. Two explanations arise. First, PM proteins could, via a specific interaction, be bound to a surrounding structure in the vicinity of the PM. For instance, PM-anchored protein A could bind to B which is a non-diffusible object or itself attached to a non-diffusible object, consequently limiting protein A diffusion. Alternatively, or in addition, PM proteins might have constrained mobility due to steric hindrance. In other words, protein lateral mobility is restricted due to crowding with other PM proteins that themselves are involved in interactions with other cellular constituents. Both explanations have been demonstrated for plant cells. In the case of Arabidopsis Formin1 (AtFH1), low lateral mobility is due to a specific interaction between the extracellular domain of the protein and a cell wall component, most likely mediated through an extensin binding motif of AtFH1 (Martinière et al., 2011). Low lateral mobility of other PM proteins might be the result of direct interactions with the cell wall or the cytoskeleton. This is perhaps the case for the WAK protein family that interact with oligogalacturonides of the cell wall (Steinwand and Kieber, 2010). Similarly, it is tempting to think that families of RLKs (receptor-like kinases), which are involved in cell wall-sensing and have large extracellular extensions, are directly linked to cell-wall constituents (for review see: Hématy and Höfte, 2008).

As stated earlier, compared to animal cells, many plant PM proteins have far smaller lateral mobilities. Nevertheless, animal PM proteins rarely exhibit free diffusion behavior. It is, in fact, common for them to have substantially altered motion. In the past 40 years, the fluid mosaic model proposed by Singer and Nicolson (1972), in which membrane components are uniformly distributed, has slowly evolved to a model in which membranes are composed of a multitude of microdomains (Engelman, 2005; Nicolson, 2013). Microdomains are maintained over time either by a heterogeneity in PM composition, e.g., as lipid microdomains, or by corrals formed of cytoskeletal elements in close proximity to the PM which limit movement of PM proteins

Table 1 | Mobile fraction of proteins evaluated by FRAP.

Constructs	Expression system	Mobile fraction %	Time of observation	References
GFP-Lti6b	Stable line p35S	76	120 s	Kleine-Vehn et al., 2011
	Transient expression, p35S	84	60 s	Martinière et al., 2012
	Stable line p35S	96	60 s	Martinière et al., 2012
PIN2-GFP	Stable lines pPIN2	13	60 s	Feraru et al., 2011
		>20	200 s	Men et al., 2008
		14	120 s	Kleine-Vehn et al., 2011
PIN1-GFP	Stable lines pPIN1	17	120 s	Kleine-Vehn et al., 2011
PIP2;1-GFP	Stable line p35S	10	60 s	Luu et al., 2012
	Stable lines p35S	10	60 s	Martinière et al., 2012
PIP2;1-CFP	Transient expression, p35S	43	60 s	Martinière et al., 2012
PIP1;2-GFP	Stable line p35S	10	60 s	Luu et al., 2012
GFP-NPSN11	Transient expression, p35S	10	60 s	Martinière et al., 2012
GFP-AGP4	Transient expression, p35S	20	60 s	Martinière et al., 2012
GFP-StREM1.3	Transient expression, p35S	23	60 s	Martinière et al., 2012
AtFLS2-GFP	Stable lines, pFLS2	19	60 s	Martinière et al., 2012
GPa1-GFP	Transient expression, p35S	79	60 s	Martinière et al., 2012
AtFH1-GFP	Transient expression, p35S	18	60 s	Martinière et al., 2012
YFP-AtSYP121	Transient expression, p35S	23	60 s	Martinière et al., 2012
At1g14870-GFP	Transient expression, p35S	36	60 s	Martinière et al., 2012
At3g17840-GFP	Transient expression, p35S	58	60 s	Martinière et al., 2012
BOR1	Stable lines	<40	20 min	Takano et al., 2010
mCitrin-NIP5;1	Stable lines	<50	20 min	Roppolo et al., 2011
GFP-KNOLLE	Stable lines, pKNOLLE	>60	10 min	Boutté et al., 2010
CASP1-GFP	Stable lines, pCASP	<15	20 min	Roppolo et al., 2011

which project into the cytoplasm (Tomishige et al., 1998). In both cases, these structures constrain lateral mobility of PM proteins. Very well documented examples show a partitioning of proteins between microdomains in, e.g., raft-, and non-raft fractions, and that microdomain organization of membrane proteins can be linked to signal transduction mechanisms (review in Simons and Ikonen, 1997; Simons and Gerl, 2010). PM proteins can exhibit various types of lateral mobility. In Li et al. (2011), fluorescent variants of GFP-PIP2;1 have been described with three motion modes: Brownian, directed, and restricted (reviewed in Owen et al., 2009). Brownian motion is typical for objects with random trajectories. Directed motion means that particles are moved via energetic processes such as in the case of myosin-mediated movement along the actin cytoskeleton. Finally, restricted motion happens when particles are confined in a small area of the membrane such as within a lipid microdomain. Interestingly, lateral mobility of GFP-PIP2;1 seems to involve all three types of motion. It is known that some minutes after addition of a salt stress, PIP proteins undergo rapid and quantitative endocytosis (Luu et al., 2012). Examination of the early events in this process found that PIP2;1 has a tendency to be restricted in its mobility. When salt treatment was combined with drug treatments to alter trafficking, PIP2;1 changed from being immobile to restricted motion and then was endocytosed. This result suggests that, as in animal cells, plant PMs have microdomain organization, e.g., lipid microdomains and/or corralling cytoskeletons which modulate protein mobility (for review see: Malinsky et al., 2013).

Lipid microdomains in plant cells have been biochemically characterized for several species including *Arabidopsis thaliana* (for review Simon-Plas et al., 2011). These microdomains can partition proteins within the plane of the membrane and show a general enrichment for proteins involved in signaling, cell trafficking and cell-wall metabolism (Borner et al., 2005; Keinath et al., 2010). Interestingly, in plant-pathogen interaction, the leucine-repeat-rich receptor kinase FLS2 is recruited into detergent insoluble fraction (DIM) upon elicitation with flg22 (Keinath et al., 2010). This suggests a model in which lipid partitioning plays a role in lateral mobility of plant PM proteins.

A cell's extracellular matrix (ECM) is also an important feature in regulating protein lateral mobility. Research in yeast has shown that the periplasm and the cell wall both modify lateral mobility of lipid probes (Greenberg and Axelrod, 1993). More recently, an extensive work on yeast PM microdomains has shown that membrane micro-organization is perturbed by loss of the cell wall (Spira et al., 2012). Similarly, in nerve cells, the ECM hinders lateral mobility of glutamate receptors (Frischknecht et al., 2009). In plant cells, outward turgor pressure forces the PM to be very tightly appressed to the cell wall. A recent study has show that this intimate connection affects protein lateral mobility (Martinière et al., 2012). Sets of artificial proteins were used to describe the influence of the cell wall mechanism on lateral mobility. Proteins with amino acids projecting into the outer phase of the membrane or into the extracellular space had a low lateral mobility which increased if the cell wall was perturbed or removed (Martinière et al., 2012). Consequently, these

results suggest that the plant cell wall, and by extension the continuum between the PM and the cell wall influences protein lateral mobility. This regulation of protein lateral mobility could play a role in myriad cellular processes. For instance, in root tip cells, the polar localization of PIN2 disappeared under plasmolysis treatment or when a weak cell wall digestion was performed (Feraru et al., 2011). The likely explanation for this observation is that the cell wall restricts lateral mobility of PIN2 and consequently helps maintain its polarized localization in root epidermal cells.

PROTEIN IMMOBILITY IN THE PM AND ITS CONSEQUENCES

The relative immobility of most plant PM proteins needs to be looked at in context. Mobile fractions generated from FRAP data are always done on small time scales from seconds to tens of minutes. This time scale undoubtedly has significance in terms of signal transduction, but is maybe less meaningful for longer process during development. However, the polarized localization of auxin transporters such as PIN1 or PIN2 is, at least in part, based on low lateral mobility within the PM. Indeed, only the additive effect of polarized exocytosis and endocytosis coupled with a low lateral mobility allows model to predict with accuracy the localization of PIN proteins (Kleine-Vehn et al., 2011). As a consequence, low lateral mobility of proteins acts in polarized localization of auxin and, therefore, participates in root development.

Recent findings about the Casparian strip are also meaningful in terms of lateral mobility of proteins within the PM. The Casparian strip is a cell wall barrier made in part of suberin and lignin surrounding the endodermis tissue in plant roots (review in Geldner, 2013). This barrier limits diffusion of water and ions between outer layers of the root and its vascular tissue. Interestingly, this barrier also stops totally the diffusion of lipids within the PM of endodermis cells (Alassimone et al., 2010). The molecular processes involved in Casparian strip formation are beginning to be understood. CASP proteins which are localized at first everywhere within the PM are later found only where the Casparian strip will be formed (Roppolo et al., 2011). Strikingly, in terms of lateral mobility, CASP proteins are relatively more mobile at early stages than in later stages of development. This suggests that in the region where the Casparian strip will be formed, CASP proteins are anchored by an unknown mechanism that might include interaction with the cytoskeleton or the cell wall (Roppolo and Geldner, 2012). This example serves to illustrate again the importance of protein lateral mobility in regulation of development processes and clearly show that plant can modify their cell wall composition to influence on diffusion of proteins within their PM.

A mechanism for regulation of cellular process such as signal reception is "control by change in location" (Malinsky et al., 2013). In others words, membrane proteins move laterally to effect activation of a signaling mechanism. This phenomenon has recently been described in the cases of the ammonium transporter AMT and the SAL3/CPIK23 complexes (explained in detail earlier). It is tempting to extrapolate these findings to other signaling cascades, especially the case of flagellin signaling, it is know that FLS2, the receptor for flg22, has its lateral mobility restricted upon interaction with its ligand (Ali et al., 2007). This again suggests an interconnection between dynamic partitioning in the PM and signal transduction mechanisms, even if formal proof is still missing.

CONCLUSION AND FUTURE PROSPECTS

FRAP approaches have revolutionized the study of membrane protein dynamics in living cells. These techniques make possible the study of membrane structure and promise to be useful in elucidation of signaling mechanisms.

Many PM proteins have very low lateral mobility which suggests a high degree of membrane organization and a natural tendency of proteins to groups themselves in clusters, e.g., KAT1, StREM3.1, PIN2, AMT1.3, and AtFlot1 (Sutter et al., 2006; Raffaele et al., 2009; Kleine-Vehn et al., 2011; Li et al., 2012; Wang et al., 2013). Bioimaging not only lets us study protein dynamics and association, but now gives us accessible tools for studying protein-protein interactions. Receptor-mediated signaling mechanisms should be observable by combining FRAP techniques with fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM). In FRET, interacting proteins become visible by alterations in their fluorescence emission and this is observable as diffusion is monitored via FRAP.

Super-resolution microscopy techniques such as stimulated emission depletion (SIM), structured illumination (STED) and photoactivated localization microscopy (PALM) are capable of resolving objects 50-70nm in size, far below the current limit for light microscopy of 200-300nm. Coupled with total internal reflection fluorescence microscopy (TIRF), its allow studying the dynamics of individual molecules. This single-molecule tracking technique has been employed to study movement of the protein in plant PM (Li et al., 2011; Martinière et al., 2012). Ongoing work in our labs will seek to combine FRAP for measurement of protein dynamics with higher resolution techniques that will allow finer-scale dissection of the mechanisms involved in membrane transport and pathogen response.

Finally, one of the main findings of our recent work was that the cell wall interacts with and stabilizes PM proteins. We are now engaged in trying to elucidate the mechanism of cell wall—PM protein interaction. One of the approaches to this problem will be to study dynamics of PM proteins in cell-wall mutant backgrounds that are altered in, or lacking different cell-wall components such as pectin and cellulose. The cell-wall may turn out to have functions in cell signaling that we do not yet appreciate.

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Plasma membrane partitioning: from macro-domains to new views on plasmodesmata

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Compartmentalization of cellular functions relies on partitioning of domains of diverse sizes within the plasma membrane (PM). Macro-domains measure several micrometers and contain specific proteins concentrated to specific sides (apical, basal, and lateral) of the PM conferring a polarity to the cell. Cell polarity is one of the driving forces in tissue and growth patterning. To maintain macro-domains within the PM, eukaryotic cells exert diverse mechanisms to counteract the free lateral diffusion of proteins. Protein activation/inactivation, endocytosis, PM recycling of transmembrane proteins and the role of diffusion barriers in macro-domains partitioning at PM will be discussed. Moreover, as plasmodesmata (PDs) are domains inserted within the PM which also mediate tissue and growth patterning, it is essential to understand how segregation of specific set of proteins is maintained at PDs while PDs domains are smaller in size compared to macro-domains. Here, we will present mechanisms allowing restriction of proteins at PM macro-domains, but for which molecular components have been found in PDs proteome. We will explore the hypothesis that partitioning of macro-domains and PDs may be ruled by similar mechanisms.

Keywords: domains partitioning, lateral diffusion, GTPases, PIN proteins, plasma membrane recycling, endocytosis, lipids, casparian strip

INTRODUCTION

A fascinating feature of living organisms is their ability to compartmentalize functions which from the sub-cellular level organize the entire organism. Similarly, to the specialization of intracellular functions in diverse organelles inside the cell, lateral compartmentalization of the plasma membrane (PM) organizes cell functions by spatially restricting interactions between specific sets of proteins and between proteins and specific membrane lipids to defined areas of the PM. Large scale domains segregation (e.g., several micrometers macro-domains) within the PM (basal, apical, outer lateral, and inner lateral membranes) is the basis for cell polarity which in turn will determine growth patterns in the whole organism. Plants have the ability to reorient the pattern of their body plan throughout the entire life span and are thus highly flexible in modulating cell polarity and segregating domains at the PM. Although our understanding of the mechanisms involved in lateral heterogeneity of the PM remains fragmented, recent progress has been made in various model systems. Here, we will review and discuss sub-cellular and molecular mechanisms which take an active part in lateral segregation of macro-domains within the PM. In a first part of this review we will describe a protein activation/inactivation mechanism through which polar domains, established at the tip of plants pollen tube cells and root hair cells, conduct polar tip growth of the cell. In a second part, we will focus on regulations acting on recycling and defined endocytosis at PM polar domains and how that is restricting lateral mobility of proteins at specific domains at PM. In a third part we will succinctly present membrane lipids as major regulators of PM macro-domains partitioning. In a fourth part, we will present recent progress made in the comprehension of the role of diffusion barriers, such as the endodermal casparian strip, and the extracellular matrix in segregating macro-domains at the PM. At last we will discuss the specific case of smaller scale PM domains than macro-domains but which function is crucial in tissue identity and patterning, e.g., the plasmodesmata (PDs). We expect that mechanisms known from other models than PDs, but for which molecular elements have been found in the plasmodesmal proteome, could help understanding proteins segregation at PDs.

REGULATION OF PROTEIN ACTIVATION PLAYS A ROLE IN LATERAL SEGREGATION OF PROTEINS AT THE PM

Guanosine triphosphate (GTP)-binding proteins are involved in determining specificity of membrane fusion and fission events. Therefore, lateral segregation of these proteins at the PM must be under fine tuned control to ensure that specific trafficking events act at defined spots. Lateral segregation of GTP-binding proteins is partly regulated through activation and inactivation of these GTP switches. Guanine nucleotide exchange factors (GEFs) catalyze the conversion of the GTP-binding protein from the inactive guanosine diphosphate (GDP)-bound form to the active GTP-bound form. Conversely, GTPase-activating proteins (GAPs) stimulate hydrolysis of GTP to GDP. Moreover, binding of guanosine nucleotide dissociation factors (GDIs) to the inactive GDP-bound form prevents GDP to GTP exchange. To be activated, proteins bound to GDP must be released from their GDI inhibitor through action of GDI displacement factors. Conversion in the active state favors interactions with downstream effectors involved in diverse

cellular functions such as polar growth for example (Molendijk et al., 2001, 2004; Vernoud et al., 2003). Some plant RHO-type GTP-binding proteins have been shown to preferentially locate in a polar fashion in polarized tip growing cells such as pollen tubes and root hairs (Lin et al., 1996; Jones et al., 2002). Both GDI and GAPs proteins have been shown to be involved in the polar localization of RHO proteins at the tip of apically growing cells. In *Arabidopsis* root hairs, the RHO protein ROP2 is polarized to the tip of the cell through a mechanism in which the GDI protein SUPERCENTIPEDE1 (SCN1)/AtRHO-GDI1 plays a major function (Carol et al., 2005). This illustrates the role of the inactivation of RHO in the regulation of its activity and its restricted polarized localization. Consistently, in tobacco pollen tubes, the GDI protein NtRHO-GDI2 and the GAP protein NtRHO-GAP1 spatially restricts the RHO-type GTP-binding protein NtRAC5 activity to the tip of pollen tubes (Klahre and Kost, 2006; Klahre et al., 2006). In this model, NtRHO-GAP1 localizes at the lateral PM close to the tip of the pollen tube but is excluded from the very tip where active membrane-bound NtRAC5 is found (Klahre and Kost, 2006). Lateral diffusion of active NtRAC5 to lateral PM would then be counteracted by inactivation of NtRAC5 which would then dissociate from the PM. However, this mechanism is not a general mechanism since in *Arabidopsis* pollen tubes the RHO-GAP protein REN1 localizes to the tip of pollen tube where the RHO-type GTP-binding protein ROP1 is present (Hwang et al., 2008). In this model, when ROP1 reaches a critical threshold level at the PM, REN1 initiates a negative feedback loop to inactivate ROP1 which would be removed from the membrane. The cyclic nature of this phenomenon results in an oscillatory tip growth (Hwang et al., 2008). If lateral segregation of membrane associated GTP-binding proteins is regulated through GTP/GDP switch, this mechanism however, does not explain how intrinsic transmembrane proteins can be confined to specific domains of the PM.

ENDOCYTOSIS AND RECYCLING ARE INVOLVED IN SPATIAL SEGREGATION OF PROTEINS AT THE PM BY RESTRICTING LATERAL DIFFUSION

Lateral segregation of auxin carriers in the PM of *Arabidopsis* roots is the most extensively studied model to understand how cell polarity of transmembrane proteins is established and maintained in distinct PM macro-domains such as apical, basal, inner lateral, and outer lateral membranes. Polar localization of the auxin efflux carriers PINs at the basal membrane greatly relies on endocytosis and PM recycling. PIN recycling has been shown to involve ADP-ribosylation factor (ARF) activation through the ARF-GEF GNOM which localizes to recycling endosomes which have yet to be structurally defined (Steinmann et al., 1999; Geldner et al., 2003; Richter et al., 2007). Interestingly, another ARF-GEF, BEN1, has been shown to act in PIN recycling at early endocytic compartments distinct from GNOM-labeled endosomes pointing out a differential regulation on PIN recycling (Tanaka et al., 2009). Additionally, ARF inactivation through the ARF-GAP SCARFACE (SFC)/VAN3 also plays a crucial function in the PM recycling of PINs (Sieburth et al., 2006). Targets of ARF-GEF and ARF-GAP are not well described although it is known that the ARF-GEF BIG3 interacts *in vitro* with ARF1-A1C protein required for BFA-sensitive PM recycling of PIN proteins (Nielsen et al., 2006; Tanaka et al., 2014). It is thought that ARF-GEF membrane recruitment to specific compartments is partly regulated through the action of RAB proteins. Consistently, it has been shown that the RAB-A1b and RAB-A1c proteins are involved in lateral segregation of PINs proteins through PM recycling (Qi et al., 2011; Feraru et al., 2012). Moreover, effector elements of RAB molecular switches are also involved. For example, subunits of the exocyst vesicle tethering complex, thought to be RAB effectors and to act in polarization of exocytosis in yeast and animals, are involved in the recycling of PIN proteins (Drdova et al., 2013). Together with PM recycling, computer simulations have suggested that PIN polar domain is maintained through the occurrence of a spatially defined clathrin-mediated endocytic area at the circumventing edges of the polar domain (Kleine-Vehn et al., 2011). During endocytosis of PIN proteins, clathrin-coated vesicles (CCVs) form at the PM and loading of cargoes in these vesicles is selectively occurring via clathrin adaptor complexes while fission of CCVs from the PM is promoted by dynamin-related proteins (Dhonukshe et al., 2007; Fujimoto et al., 2010; Kitakura et al., 2011; Mravec et al., 2011; Fan et al., 2013). Additionally, RHO-type GTP-binding proteins ROP2 and ROP6 are master regulators of clathrin-dependent endocytosis pointing out again the central importance of GTP-binding proteins in lateral segregation of proteins (Chen et al., 2012; Lin et al., 2012; Nagawa et al., 2012).

MEMBRANE LIPIDS ARE MAJOR REGULATORS OF PM MACRO-DOMAINS PARTITIONING

Several classes of lipids have been shown to be implicated in partitioning of macro-domains at PM. Bulk sterols are membrane lipids and key elements in endocytosis-mediated establishment of the auxin efflux carrier PIN2 polar domains at the PM during post-cytokinesis (Men et al., 2008). Recently, it has been shown that not only bulk sterols are involved in polar auxin transport but sterol biosynthetic intermediates also act in this process pointing out the likely high complexity of sterols in regulation of polarity of auxin carriers (Mialoundama et al., 2013). Additionally, sterol-mediated endocytosis is involved, together with clathrin-mediated endocytosis, in restricting lateral diffusion of a major player of vesicle fusion, the syntaxin KNOLLE, from the division plane to lateral membranes during cytokinesis (Boutté et al., 2010). Accordingly, clathrin light chain and one of its interacting partners, T-PLATE, accumulate at the cortical division zone of lateral PM juxtaposing the division plane (Van Damme et al., 2011). These studies suggest that spatially defined sterol- and clathrin-mediated endocytosis regulate lateral segregation of proteins to the division plane during cytokinesis. Very-long-chain-fatty-acids (VLCFAs) present in phospholipids and sphingolipids pools were also shown to be important in endocytosis and restriction of the KNOLLE macro-domain at the cell plate during cytokinesis (Bach et al., 2011). Indeed, it was also shown previously that an isoform of the phospholipase D, which cleaves the phosphodiester bound of a phospholipid and consequently produces the phosphatidic acid (PA), and an isoform of the phospholipase A, which cleaves a fatty acyl chain of a phospholipid, are critical for endocytosis and recycling at PIN2 PM macro-domain (Li and Xue, 2007; Lee

et al., 2010). Sphingolipids are also known to be involved in polarized PM distribution of PIN although the precise role played by sphingolipids in cellular mechanisms, e.g., secretory and endocytic pathways, still has to be determined (Roudier et al., 2010; Markham et al., 2011). Lastly, phosphoinositides, although representing a minor class of phospholipids, also appear as a crucial class of lipids involved in segregation of PIN polar domains at the PM. This is exemplified recently by the role of phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P_2] in clathrin-mediated PIN1 and PIN2 polar domains segregation at the PM (Ischebeck et al., 2013). Additionally to endomembrane trafficking events and their regulation, the extracellular environment of the cell has also to be considered in segregating domains at PM. Indeed, the cell wall plays an integral role in the framework modulating cell polarity and lateral diffusion of proteins (Feraru et al., 2011; Martinière et al., 2012).

MECHANICAL CONSTRAINTS APPLIED BY THE EXTRACELLULAR MATRIX INFLUENCE LATERAL SEGREGATION OF PROTEINS IN PM

Eukaryotic multicellular organisms such as animals and plants are using the extracellular matrix properties to segregate domains within the PM. In polarized animal cells, junctional complexes (including tight junctions and adherens junctions) are known to be involved in restricting the movement of membrane proteins and lipids (van Meer and Simons, 1986; Laval et al., 2008; Renner et al., 2012). Junctions are constituted of a dense scaffolded arrangement of proteins included in the extracellular matrix made of polysaccharides. In plants, the cell wall is also constituted of polysaccharides such as cellulose (polymer of D-glucose through β-1,4-linkage), hemicelluloses (xyloglucan and xylan, a polymere of xylose), pectins (homogalacturonans and substituted galacturonans), and lignin (phenolic polymer). A good example of PM domains segregation mediated by cell wall specialization is the casparian strip, a belt which transversally surrounds endodermal root cells in an anticlinal orientation and which is constituted of lignin in the early stages, although suberin could also be constitutive at later stages (Alassimone et al., 2010; Naseer et al., 2012). The casparian strip is a diffusion barrier which functional features resemble tight and adherens junctions of animal polarized epithelia although protein and extracellular matrix composition is different (Roppolo et al., 2011; Naseer et al., 2012). The casparian strip is splitting endodermal cells in distinct lateral PM domains, an outer domain facing the periphery of the root and an inner domain facing the central part of the root in which distinct proteins are found (Alassimone et al., 2010). The PM domain underlying the casparian strip is located at the crossroad of the outer and inner lateral domains and contains casparian strip domain proteins (CASPs) which constitute a dense scaffold and are involved in focalizing lignin deposition during casparian strip formation (Roppolo et al., 2011; Lee et al., 2013). CASPs proteins and the specialized cell wall region of the casparian strip form a diffusion barrier for proteins and lipids (Alassimone et al., 2010). Segregation of lateral PM domains allows endodermal cells to select nutrients and minerals as exemplified with the boron influx transporter NIP5;1 which localization is constrained to the outer lateral domain and the boron efflux transporter BOR1 which restriction to the inner lateral domain consistently supports its role in loading xylem cells of root central cylinder (Alassimone et al., 2010; Takano et al., 2010). Additionally, localization of other carriers such as auxin carriers is also differentially regulated in endodermal cells in which the casparian strip is present. The auxin efflux carrier PIN2 is found to be localized at the basal membrane in protoendodermal cells, at the apical membrane in elongating endodermal cells and finally at the inner lateral membrane in differentiated endodermal cells in which the casparian strip is in place (Alassimone et al., 2010). This cell differentiation stage-dependent relocation of PIN2 exemplifies that regulation of domain segregation within the PM can be different when a diffusion barrier such as the casparian strip is present.

The cell wall is also known to be involved in membrane domains segregation in other cell types than differentiated endodermal cells. In BY2 cells and *Arabidopsis* meristematic root cells, it has been shown that PIN polarity is lost when the cell wall is progressively chemically removed (Boutté et al., 2006; Feraru et al., 2011). Moreover, mutants deficient in cellulose composition display PIN polarity defects at the PM of epidermal cells (Feraru et al., 2011). Interestingly, a gentle plasmolysis which preserves the cell wall results in retraction of the PM but retains PM connection with the cell wall at defined spots labeled with PM proteins localized in polar domains, such as the auxin efflux carrier PIN1, but not with proteins which localization is apolar (Feraru et al., 2011). This observation suggests that specific membrane attachment to the extracellular matrix could be important in lateral segregation of domains.

SEGREGATION OF PLASMODESMATA WITHIN THE PM COULD SHARE SIMILAR MECHANISMS AS FOR MACRO-DOMAINS

Plasmodesmata are specific domains of the PM organized as nanopores and which function in cell-to-cell communication is essential to virus movement, cell identity induced by movement of transcription factors from cell layer to cell layer, and patterning during lateral roots development and embryogenesis (Wolf et al., 1989; Lucas et al., 1995; Kim et al., 2005; Kurata et al., 2005; Vaten et al., 2011; Wu and Gallagher, 2012; Benitez-Alfonso et al., 2013; Tilsner et al., 2013). Hence, given their role in diverse processes, it is essential to understand how plants restrict proteins at PDs sites of the PM. Whether regulation of protein activation through GDP/GTP cycling has a function in restricting lateral diffusion of proteins at PDs remain unaddressed. However, several GTP-binding proteins are found in the *Arabidopsis* plasmodesmal proteome: proteins from the ARF family (all from the subclass A, and several ARF-like proteins), proteins from the RAB family (from subclasses A, E, and G) and proteins from the GDI and GEF families (Fernandez-Calvino et al., 2011). GTP-binding proteins of the dynamin-related family which could be involved in membrane constriction of the desmotubulus were also found and their localization could also be regulated through GTP/GDP switch. Moreover, PDs are involved in movement of viruses and a recent study suggests a role for a RAB-GAP in Bamboo mosaic virus intercellular movement (Huang et al., 2013). Alternatively and/or additionally, GTP-binding proteins could be involved in

PDs-localized endocytosis and recycling which would restrict lateral diffusion of proteins at PDs. Indeed, elements of the clathrin machinery have been identified in the plasmodesmal proteome including clathrin heavy chain, subunits of clathrin adaptor complexes, and dynamin-related proteins (Fernandez-Calvino et al., 2011). Apart from proteins, the function of membrane lipids in lateral segregation of proteins at PDs, and in PDs functioning in general, is still a determinant question to address. Similarly, the function of the cell wall in lateral segregation of proteins at PDs is still to be addressed. Interestingly, PDs are structures which retain PM close contact to the cell wall upon plasmolysis similarly to what has been described for PIN1 (Oparka, 1994; Feraru et al., 2011). Interestingly, auxin efflux carriers PIN1 and PIN7 have been found in the plasmodesmal proteome (Fernandez-Calvino et al., 2011). Hence, it is possible that specific spots where PINs labeled membranes attached to the cell wall correspond to PDs. However, it is not known whether cell wall components known to be involved in lateral segregation of polar domains, such as cellulose, play a role in PDs functioning and/or lateral segregation of domains at PDs, although cellulose synthases have been found in the plasmodesmal proteome (Fernandez-Calvino et al., 2011).

CONCLUSION

Eukaryotic cells partition the PM into distinct domains to compartmentalize cellular processes and specify functions critical in growth patterning. Polar tip growth and auxin-mediated developmental processes require segregation of polar domains at the PM. Protein activation/inactivation, PM recycling and endocytosis, diffusion barriers, and the extracellular matrix are known to be involved in domain partitioning during polar tip growth or in auxin carriers polar positioning at the PM. PDs which function is crucial in tissue patterning also requires partitioning of proteins. This specifies domains and segregate processes although mechanisms through which this happens have yet to be discovered. Future studies might reveal whether and how plasmodesmal-localized activable GTP-binding proteins, and their regulators, are involved in lateral segregation of proteins involved in plasmodesmal-related processes. Importantly, whether endocytosis and recycling are also involved in lateral segregation at PDs should be investigated. Finally, as PDs can be viewed as cell junctions, future studies would need to combine genetics, biochemistry, and cell biology to decipher the role of cell wall components in the function and lateral segregation of proteins at PDs.

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Crystallization around solid-like nanosized docks can explain the specificity, diversity, and stability of membrane microdomains

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To date, it is widely accepted that microdomains do form in the biological membranes of all eukaryotic cells, and quite possibly also in prokaryotes. Those sub-micrometric domains play crucial roles in signaling, in intracellular transport, and even in inter-cellular communications. Despite their ubiquitous distribution, and the broad and lasting interest invested in those microdomains, their actual nature and composition, and even the physical rules that regiment their assembly still remain elusive and hotly debated. One of the most often considered models is the raft hypothesis, i.e., the partition of lipids between liquid disordered and ordered phases (Ld and Lo, respectively), the latter being enriched in sphingolipids and cholesterol. Although it is experimentally possible to obtain the formation of microdomains in synthetic membranes through Ld/Lo phase separation, there is an ever increasing amount of evidence, obtained with a wide array of experimental approaches, that a partition between domains in Ld and Lo phases cannot account for many of the observations collected in real cells. In particular, it is now commonly perceived that the plasma membrane of cells is mostly in Lo phase and recent data support the existence of gel or solid ordered domains in a whole variety of live cells under physiological conditions. Here, we present a model whereby seeds comprised of oligomerised proteins and/or lipids would serve as crystal nucleation centers for the formation of diverse gel/crystalline nanodomains. This could confer the selectivity necessary for the formation of multiple types of membrane domains, as well as the stability required to match the time frames of cellular events, such as intra- or inter-cellular transport or assembly of signaling platforms. Testing of this model will, however, require the development of new methods allowing the clear-cut discrimination between Lo and solid nanoscopic phases in live cells.

Keywords: membrane microdomains, amorphous materials, crystal seeds, crystallization, membrane transduction, rafts

INTRODUCTION: FROM SINGER AND NICOLSON TO THE RAFT HYPOTHESIS AND BEYOND

Biological membranes are a fundamental component of all living cells. As originally proposed by Singer and Nicolson (1972), the matrix of biological membranes is a lipid bilayer, comprised of several thousand different lipid species, with their hydrophilic heads facing the outside, and their hydrophobic moieties toward the core of the bilayer. Membranes also contain a large variety of proteins, which are less numerous than lipids, but usually amount to more than half of membranes' dry weight.

The existence of clusters of certain proteins was already described in Singer and Nicholson's original paper (Singer and Nicolson, 1972), including for the H2 antigens which had been monitored by Frye and Edidin (1970), in their seminal study of heterokaryons, through which they established the fluid nature of the plasma membrane (PM). The existence of membrane domains, and even of solid domains, was clearly proposed in the updated fluid mosaic model published by Nicolson (1976) just 4 years later. Although those articles were each cited many hundreds of times, for the 20 years that followed, the existence of lateral heterogeneities in biological membranes was dismissed by many scientists in the field. After the observation of sub-cellular lipid sorting by van Meer et al. (1987), a turning point was the formulation of the raft hypothesis by Simons and Ikonen (1997), which became very popular among biophysicists and cell biologists. In short, this hypothesis proposed a view of membrane organization based on the existence of "rafts" enriched in sphingolipids and cholesterol, which would ensure the specific transport of particular proteins between cellular compartments, and direct the assembly of signaling platforms. One of the tenets of the raft hypothesis was that rafts were resistant to solubilization at 4°C with a non-ionic detergent such as Triton X-100. Although the approach of isolating detergent resistant membranes (DRMs) had the undisputable advantage of being based on experimental observations, this convolution of the theoretical notion of rafts with the operational definition of DRMs has generated numerous misconceptions and over-interpretations that have already been summarized by others

for plants and fungi (Malinsky et al., 2013) and for animal cells (Quinn, 2010).

Before the formulation of the raft hypothesis, the idea that microdomains exist in biological membranes and may be related to the different lipid phases found in model membrane systems had already been discussed for many years (Stier and Sackmann, 1973; Nicolson, 1976; Karnovsky et al., 1982; Welti and Glaser, 1994). In initial studies, mostly based on the photophysical properties of fluorescent probes, some domains were labeled as "solid/fluid," but it is now known that these putative solid domains would be better described as liquid ordered (Lo; Bastos et al., 2012). This Lo state is formed due to the ability of membrane-active sterols such as cholesterol to provoke an intermediate state of lipid bilayers, which are still completely fluid, but are stiffer, thicker, and less permeable to water than when in liquid disordered phase (Ld), i.e., completely fluid (Dufourc, 2008). Lipid bilayers containing sterols can still harbor gel phase domains, which will be described below, albeit at lower temperatures and lower sterol concentrations, and the term solid ordered (So) is then often used to describe them.

Despite the intense interest in studying membrane microdomains, their precise nature – and even the physical rules (in other words the thermodynamic principles) that govern their assembly – still remains elusive and hotly debated. To date, the most commonly held view is that the formation of rafts corresponds to a situation of coexistence of Lo and Ld phase domains (see London, 2005 for review and historical account). Whilst it is possible to observe the coexistence of Ld and Lo phases in many membrane model systems, the relevance of such domains to the microdomains in membranes of live cells is still highly disputed (Elson et al., 2010). For example, in synthetic membranes where Ld and Lo domains coexist, most proteins – even those considered as raft-specific proteins – partition preferentially into the Ld phase (Dietrich et al., 2001b; Bacia et al., 2004; Kahya et al., 2005; Nikolaus et al., 2010). This is also true of many other molecules such as fluorescent probes (de Almeida et al., 2009) and drugs (Custodio et al., 1991).

The real-life structure of membranes is undoubtedly much more complex than a simple dichotomy between disordered and ordered domains. Specifically, the simplistic Ld/Lo partition model cannot provide a satisfactory explanation (i) for the formation of multiple types of microdomains within the same cell, which may even often occur simultaneously; (ii) for the recent evidence that most of the PM of eukaryotes is in the Lo phase, with cholesterol acting more as fluidifier and as a homogenizer rather than as a promoter of domains; (iii) for the fact that membrane microdomains are present in bacteria that lack both sphingolipids and sterols; (iv) for the occurrence of domains in solid or gel phase in eukaryotes under physiological conditions.

In the following pages, we will address these issues successively, and propose an alternative mechanism for the formation of highly selective and/or stable microdomains by a process of crystalline recruitment into membrane "docks" seeded by specific proteins/lipids. Membrane docks in a solid-like state may be an important seed for the formation of highly specific and/or stable membrane microdomains, but the detection of such nano-structures is a technological challenge and, maybe more importantly, will require that scientists accept the possibility of their existence.

THE DIVERSITY OF MICRODOMAINS

The PMs of eukaryotes contain several types of microdomains; examples include not only the "standard" rafts, but also caveolae (Parton and Simons, 2007), the recently described "heavy" rafts that may be involved in T cell signaling (Hrdinka et al., 2012), cytoskeleton-dependent sphingolipid domains (Kraft, 2013), ceramide-rich platforms (Stancevic and Kolesnick, 2010), and tetraspanin-enriched domains (Yanez-Mo et al., 2009).

What follows is by no means an exhaustive review of all the different types of microdomains, but a collection of a few selected examples that either demonstrate, or are particularly suggestive of the simultaneous occurrence of different domains in the membranes of eukaryotic cells that cannot possibly be explained by Ld/Lo phase separation alone. Other examples will refer to domains that do not fit the standard definition of a raft, i.e., a microdomain enriched in sterols and sphingolipids.

In T cells, different gangliosides are needed for activation of various subsets of T cell: GM3 for CD4+ T cell activation and GM1 for CD8+ T cells (Nagafuku et al., 2012). These authors attributed this finding to the existence of different types of functional raft domains containing specific ganglioside species in each T cell subset.

In HEK cells, the simultaneous existence of multiple types of membrane domains was recently suggested by the distinct localizations of two raft-associated proteins in different regions of the PM, as revealed by total internal reflection microscopy (Asanov et al., 2010).

Whereas "rafts" are defined as cholesterol and sphingolipid-enriched domains, sphingolipid domains are not necessarily enriched in cholesterol: fluorescence lifetime imaging microscopy revealed cholesterol-independent, sphingolipid-enriched microdomains containing signaling molecules in the same membranes as cholesterol-dependent microdomains (Hofman et al., 2008). More recently, compelling evidence for the existence of cholesterol-independent sphingolipid domains has come from chemical mapping studies using isotope-labeled sphingolipids and cholesterol in fibroblasts. Whereas cholesterol was homogeneously distributed, this approach revealed the presence of sphingolipid microdomains, which disappeared after cytoskeleton disruption (Frisz et al., 2013a,b). Further evidence for sphingolipid domains is summarized in a recent review (Kraft, 2013).

The membrane microdomains in plants and fungi have features that differentiate them from what the mammalian research community calls rafts (Malinsky et al., 2013), possibly related to the fact that plants and fungi have cell walls. These domains are in general more stable than those of mammalian cells - they can last for the duration of a cell cycle, and since they are usually observed by conventional fluorescence microscopy, they are also much larger than the few nanometers usually attributed to lipid rafts. A systematic study of the yeast *Saccharomyces cerevisiae* has revealed that the PM is organized into patches and networks of numerous domains containing specific subsets of proteins with similar transmembrane domains (Spira et al., 2012). This organization of yeast membrane compartments/membrane microdomains correlates with their lipid composition and is actively maintained by the cell through energy-consuming processes (Spira et al., 2012; Malinsky et al., 2013).

Although lipid phases most likely contribute to the formation of domains, these various examples illustrate that the lateral organization of biological membranes cannot rely simply on a dichotomy between Ld phases, enriched in glycerophospholipids with unsaturated lipid chains, and Lo phases, enriched in sphingolipids and sterols. If Ld and Lo phases do not suffice to explain how lipids organize the variety of membrane domains, what does? Proteins undoubtedly play a central role in orchestrating the organization of biological membranes but, given the pivotal role of sterols in influencing the phase behavior of lipid bilayers, the following section will first explore how the biophysical properties of sterols might contribute to organizing membrane domains.

THE ROLE OF STEROLS

Sterols bring order to the fluid phase of biological membranes, which is linked to their capacity to increase membrane impermeability to water and small ions, as well as their rigidity and solidity (Haines, 2001; Hauss et al., 2002). Yet sterols can also act as solvents and homogenizers: when above a certain threshold amount, they prevent the appearance of solid domains in a large variety of lipid mixtures and facilitate the interactions between lipids with very disparate melting temperatures (T_m; some below 0°C and others as high as 60°C), with some paradigmatic examples given below.

When most hydrated phospholipids and sphingolipids are heated, they usually undergo several phase transitions, the most important of which is from a solid-like state (also called gel) to a liquid-like state (the fluid phase). Methods such as differential scanning calorimetry can be used to determine the temperature of this gel–fluid phase transition of the bilayer, which is also called the main transition temperature T_m, or melting temperature. The main transition owes this designation to the fact that it is the most cooperative and the one with the highest associated enthalpy change for phospholipids. Although different sterols populate the PM of mammalian, fungal, and plant cells, when present in a sufficiently high molar fraction [around 30 mol% in the case of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)], they all have the ability to prevent the abrupt gel–fluid transition by provoking the formation of a single Lo phase (**Figures 1A–C**). Conversely, at lower concentrations, those sterols promote the coexistence of So/Lo phases below the temperature of three phase coexistence (which is slightly below T_m), and the coexistence of Lo/Ld above this temperature. Thus, any lipid with a sufficiently high T_m value can, in principle and under certain conditions, form gel domains in the context of a biological membranes [e.g., phospholipids with unusually long and saturated acyl chains, and many (glyco)sphingolipids]. Moreover, it has been established that sphingolipids and glycerophospholipids can form stoichiometric complexes with crystalline characteristics and very high thermal stability (Quinn, 2010).

Pioneering studies of domain imaging in eukaryotic biological membranes suggested that these micro-heterogeneities should be in a fluid state (Rodgers and Glaser, 1991), and to date, the idea that domains in gel/solid/crystalline state could play physiological roles in biological membranes is rejected by most membrane biophysicists, but we contend that these views are mostly based on indirect arguments rather than on direct and solid grounds. First, it is widely perceived that "frozen" structures are not compatible with life but, as we will describe in later sections, nanoscopic structures in a gel/solid/crystalline state can remain extremely dynamic, especially over the timescales of cellular events, i.e., seconds or minutes. Second, many proteins reconstituted into liposomes with no sterols lost their activity when the bilayer was in the gel phase, i.e., when the temperature was below the T_m of the lipids (Welti et al., 1981). Such observations do not, however, rule out that solid domains could exist in live cells, and could even be involved in turning off the activity of certain proteins. Third, in isolated membranes or in liposomes prepared with lipids extracted from PMs of cells from various sources, the measured values of T_m seemed to be, in most cases, just below the growing temperature of the original cells or the physiological temperature (e.g., 37°C for human erythrocytes; Mouritsen, 1987). Such membranes do not, however, contain all the proteins that could play critical roles in regulating the state of the lipids. In fact, as mentioned below, there are many situations where large fractions of certain PM proteins are immobile in the timescale of fluorescence recovery after photobleaching (FRAP) experiments, and this stable location is important for their biological function. All together, those arguments are thus quite far from providing a definite proof that solid domains could not exist in biological membranes. However, since Singer and Nicholson's fluid mosaic model suggested that the membranes of living organisms under physiological conditions should all be in a fluid state, the discovery of the Lo/Ld immiscibility provided the grounds to assume that microdomains in biomembranes would correspond to this type of phase separation (London, 2005).

The direct observation by imaging techniques of coexisting Ld and Lo phases in giant unilamellar vesicles (GUV) generated using lipids that were either synthetic or extracted from mammalian cells (Dietrich et al., 2001a) provided an important experimental support for the lipid raft hypothesis. When the cholesterol content is low (i.e., below 10%), however, all such systems making use of lipids with very different T_m values display typical gel/fluid phase coexistence similar to those found in binary lipid mixtures (**Figures 1D–F**). Although there is not always a complete agreement regarding some of the ternary phase diagrams presented by different authors (e.g., **Figures 1E,F**), it is clear that, under the right conditions, ternary mixtures comprised of the right proportions of sterol and of lipids with high T_m and low T_m can harbor coexisting Ld/Lo regions, both as planar bilayers or as GUVs. In some instances, the co-existence of gel and Lo regions is also possible, and, more recently, the possibility of having So/Lo/Ld coexistence, even in one single GUV, has been demonstrated (**Figure 2A**; de Almeida et al., 2007).

In membranes reconstituted with lipids extracted from cells, however, one should not forget that:

(i) proteins are not present in reconstituted membranes, whereas they comprise a very significant proportion of biological membranes (often more than 50% by weight), and proteins can have organizing or rigidifying properties (Harrington et al., 2012), or the capacity to act as seeds for a liquid-solid transition (Schram and Thompson, 1997; Joly, 2004; Schram and Hall, 2004). Conversely, some proteins are also known to limit the packing ability of lipids (Aresta-Branco et al., 2011) and to decrease their average

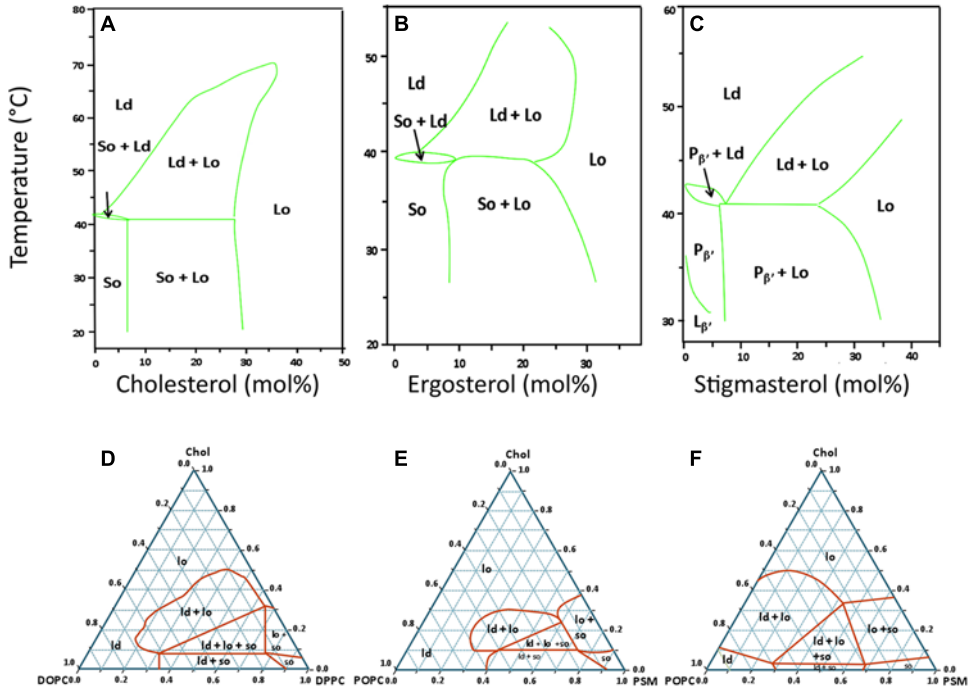

FIGURE 1 | Typical lipid phase diagrams used in the study of sterol-related membrane domains. Top row: temperature – composition binary phase diagrams for mixtures of DPPC with three different sterols **(A)** Cholesterol (animals; Sankaram and Thompson, 1991) and references therein, **(B)** Ergosterol (fungi; Hsueh et al., 2005), **(C)** Stigmasterol (plants; Wu et al., 2006). These three diagrams were selected to show that, for the same saturated lipid (DPPC), the type of phase diagram and the regions of coexistence of Ld, Lo, and/or So phases are similar for the three eukaryotic kingdoms. For panel **C**, the techniques used allowed to distinguish two types of gel phases below the T_m: the tilted gel phase ($L_{\beta'}$) and the periodic quasi-lamellar gel phase ($P_{\beta'}$). Bottom row: ternary phase diagrams for mixtures of cholesterol with two other lipids, one with a high T_m and the other with a low T_m. **(D)** Typical phase diagram for the DOPC/DPPC/cholesterol system at 24°C. This mixture is one of the best characterized systems, and the results obtained by different laboratories, even when using different techniques for phase characterization are usually in very close agreement (Veatch et al., 2004; de Almeida et al., 2007). Incidentally, it was with this mixture that the coexistence of the three phases (Ld, Lo, So) in one single GUV was experimentally demonstrated for the first time (**Figure 2A**). Panels **E** and **F** are an example of divergent phase diagrams reported by two different laboratories for a similar mixture of *N*-palmitoylsphingomyelin (PSM), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and cholesterol, at the same temperature of 23°C (**E**: Ionova et al., 2012; **F**: de Almeida et al., 2003). These two phase diagrams illustrate that there is no complete agreement for this mixture when different methods and techniques are used to detect regions of phase coexistence. This system is, however, considered to be more biologically relevant than the one on panel **D** since POPC, the low T_m phospholipid, has a saturated and an unsaturated acyl chain, and sphingomyelin is the most abundant sphingolipid in the PM of mammalian cells.

transition temperature and widen the transition (Cameron et al., 1983).

(ii) the lipid composition of reconstituted membranes may not always faithfully reflect that of the original PM they were extracted from because of possible contamination with membranes from intracellular compartments or because some of the PM lipids with very high T_m may not incorporate into the reconstituted membrane as efficiently as the more fluid ones (Ali et al., 2006; Silva et al., 2006).

Sterol contents in yeast, plants and animal PM are classically above 30 mol% of lipids (van Meer et al., 2008), which actually suggests rather strongly that most of those membranes should be in Lo phase. In agreement, several groups have indeed advocated that the PM of different types of cells is probably mostly comprised of Lo phase. For example, Owen et al. (2012) have estimated that, in a T cell line, over 75% of the PM is in Lo phase. This is not to say that the PM is necessarily completely homogenous since theoretical simulations have suggested that sterol-rich Lo phases can still display nanoscopic heterogeneities (Miao et al., 2002). Another example lies with the comparison of membrane thicknesses derived from neutron scattering, which revealed small differences between isolated putative rafts and the average thickness of the membrane (4.64 vs. 4.53 nm, respectively; Quinn, 2010). This can be taken as an indication that most of the PM is in fact in a Lo-like state, and this result somehow weakens the idea that hydrophobic mismatch between lipid bilayers and transmembrane proteins could be solely responsible for sorting proteins into different membrane domains.

Perhaps the most important message that can be taken from those various studies and phase diagrams is that, above certain sterol concentrations, all lipid bilayers will tend to be in a Lo phase over broad ranges of temperatures (**Figure 1**), including membranes containing ceramides with very high T_m (Castro et al., 2009; **Figures 2B,C**). Another very important conclusion to be drawn from all these studies is that even relatively simple mixtures display complex phase behaviors. Since the PM of an eukaryotic cell is comprised of several hundred or thousand different lipid species and many different proteins, one can thus expect that they

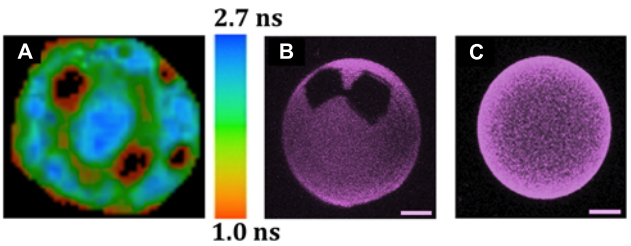

FIGURE 2 | Observation by different fluorescence microscopic techniques of lipid domain organization in GUVs and of the solubilizing properties of cholesterol. The GUVs were all labeled with Rho-DOPE (0.2 mol%), which partitions preferentially into Ld over Lo phases, and is excluded from So domains. **(A)** Coexistence of three lipid phases (Ld/Lo/So) in one single GUV, as detected by FLIM after two-photon excitation (de Almeida et al., 2007). The image corresponds to the top view of a ternary GUV DOPC/DPPC/cholesterol 11:11:3 mol:mol:mol at 24°C. The 3 phases were identified by combining the data from fluorescence intensity and lifetimes. So (black areas), Lo: intermediate intensity and mean lifetime of ∼1.7 ns (green areas), Ld: higher intensity and mean lifetime of ∼2.5 ns (blue areas). The sidebar provides the fluorescence lifetime color code. **(B,C)** The presence of 12% of *N*-palmitoylceramide (PCer) results in the formation of gels domains, even with 10% cholesterol, but such domains are not seen at 40% cholesterol, even when the amount of PCer is raised to 20 mol% (Castro et al., 2009). The precise GUV compositions are **(B)** POPC/cholesterol (9:1) with 12 mol% PCer and **(C)** POPC/cholesterol (3:2) with 20 mol% PCer. The images are 3D projections obtained from confocal sections of GUVs at 22°C (scale bar: 5 μm).

have very complex phase behaviors. Because the membranes of bacteria contain fewer lipids and no sterols, it is tempting to turn to those as an alternative model of phase behavior in biological membranes.

MICRODOMAINS IN BACTERIAL MEMBRANES

Although bacterial membranes do not contain sterols, microdomains can clearly form in the membranes of bacteria (Lopez and Kolter, 2010). For example, functional microdomains involving a flotillin homologue have been identified in the membranes of bacteria, namely *Bacillus subtilis* and possibly also in *Staphylococcus aureus* and *Escherichia coli* (Lopez and Kolter, 2010). Given that bacteria are devoid of sterols (with a few notable exceptions mentioned below), and most of them also of sphingolipids, those domains must thus clearly differ from eukaryotic rafts. In fact, bacterial membranes seem to rely more on a solid/liquid dichotomy than on an Lo/Ld partition. Multiple reports, based on a variety of approaches, have indeed documented that, although the membranes of exponentially growing bacteria are usually in a fluid phase, solid phases tend to appear as soon as the temperature is reduced, or under a variety of other conditions. The existence of gel phases in bacterial lipid membranes was first described as early as 1975, using nuclear magnetic resonance (NMR) spectroscopy to investigate the physical state of deuterated palmitic acid which had been incorporated into the membranes of *Acholeplasma laidlawii* bacteria. This revealed that, just underneath the growth temperature, a significant portion of the bacterial membrane turned to a gel state (Stockton et al., 1975). Interesting studies were later performed on the same bacterial species where the ratio of lipid saturation in the bacterial membranes was increased by feeding C14:0 saturated myristic acid to either fatty acid-auxotroph bacterial strains or to WT strains in the presence of drugs inhibiting fatty acid synthesis. It was found that, even at the normal growth temperature, ∼85–90% of the membrane lipids were in gel state, and that proteins had little effect on lipid order (Jarrell et al., 1982). Another study based on FTIR (Fourier-transform infrared spectroscopy), also using live *A. laidlawii* bacteria, showed that, at 30°C (i.e., the growth temperature), a significant percentage of lipids was in the gel phase, and that below 20°C, the membranes were entirely in gel phase, whilst very high viability was still preserved (98–99%; Cameron et al., 1983). A recent study also based on FTIR has shown that, in *Geobacter sulfurreducens*, gel phases are caused by osmotic stress or desiccation (Ragoonanan et al., 2008). Although the above studies, which are mostly based on "forced-feeding" saturated fatty acids are not entirely physiological, they do show that bacterial cells can survive and even grow containing high amounts of gel phase in their PM. In support of this, it has been estimated that *E. coli* can grow normally with as much as 20% of their membrane lipids in a gel state (Jackson and Cronan, 1978).

Although prokaryotes do not contain sterols, most of them possess various forms of branched or cyclic polyterpenoid lipids that strengthen their membranes and contribute to making them more resistant to potential toxic molecules such as alcohol, and more impermeable to water than bilayers made simply of linear lipids such as phospholipids (Ourisson et al., 1987). Among those surrogate molecules, the group of molecules known as hopanoids share many structural features with sterols. Recently, a biophysical study has shown that diplopterol, the simplest bacterial hopanoid, can interact with N-stearoyl-sphingomyelin and induce the formation of an Lo-like phase (Saenz et al., 2012).

To date, only a handful of eubacteria have been identified that have the ability to synthesize sterols, and many of those probably acquired the genes by horizontal gene transfer from eukaryotes (Hannich et al., 2011). Among those bacteria that can synthesize sterols, however, a particularly interesting phylum is that of the Planctomycetes, which carry genes for sterol synthesis that have been proposed as the likely precursors of those found in eukaryotes (Pearson et al., 2003). For a variety of reasons, Planctomycetes are increasingly perceived as the phylum that most likely gave rise to eukaryotes, presumably via an event of symbiotic fusion with an archae bacterium. For example, eukaryote-like features of the planctomycete species *Gemmata obscuriglobus* include: a large volume (3 to 5 times larger than typical bacteria such as *E. coli*); a genome of ∼8000 genes, including some for ancient tubulins for a rudimentary cytoskeleton; the lack of peptidoglycan in the cell wall; and finally the presence of endomembranes, which allow those bacteria to carry out an endocytic-like process (Devos, 2013). To date, among prokaryotes, Plantomycetes are the ones with the most developed endomembrane system. There is therefore a striking correlation between the acquisition of sterols and the capacity to harbor at least two different types of membranes, one for the outer PM and one inside the cell. Of note, in eukaryotes, whilst the PM is rich in sterol, the inner membranes, including those of the nucleus and of mitochondria, are rather akin to standard prokaryotic membranes (van Meer et al., 2008; Lippincott-Schwartz and Phair, 2010) because they harbor almost no cholesterol and must thus be much less prone

to harboring Lo phases. They will, however, remain in a fluid Ld state most of the time because they contain only minute amounts of sphingolipids and are mostly comprised of glycerophospholipids with low T_m such as PC and PE, with high proportions of unsaturated fatty acids (Fridriksson et al., 1999; van Meer et al., 2008).

Sphingolipids are much more widely distributed than sterols among prokaryotes, and meta-consensus analyses have revealed that the enzymes for sphingolipid metabolism are among the most widespread, and can be found in all three domains of life (Goldman et al., 2012). So far, however, there are no known prokaryotes that simultaneously harbor both sphingolipids and sterols (Hannich et al., 2011). Conversely, and rather strikingly, the PM of all known eukaryotes contains high amounts of both sterols (30 to 50%) and sphingolipids (ca. 15%; van Meer et al., 2008). Some eukaryotes, such as drosophilae, cannot synthesize sterols, but still need them and have to obtain them from their diet.

COEXISTING SPHINGOLIPIDS AND STEROLS: FROM PROKARYOTES TO EUKARYOTES

One broadly favored scenario for the appearance of the first eukaryote is one of a symbiotic fusion event between an archae and a eubacterium, subsequently followed by engulfment of a purple bacterium that would evolve into mitochondria (Margulis, 1996). Although it was initially suggested that the heterochiral membranes that must have resulted from the fusion of a eubacterium and an archae would be very unstable, this inherent instability has not been confirmed (Lombard et al., 2012). Worthy of note, in eukaryotes, sphingolipids are predominantly found in the extracellular leaflet (Zachowski, 1993) whereas sterols, at least in some cells, are predominantly in the cytoplasmic leaflet (Mondal et al., 2009). A particularly interesting idea put forward in this latter paper is that an important role of sterols could be related to their capacity to flip very rapidly between the two leaflets, allowing them to fill any gaps that could result from metabolism or transport events, thus resulting in a better stability of the membrane.

From a lipid-centric perspective, given the information provided in the previous paragraphs, one can be tempted to suggest that a critical event in the making of eukaryotes lied with the fusion of a sterol-containing planctomycete-like eubacterium on the one hand, and a sphingolipid-containing bacterium on the other (this latter corresponding either to the presumed ancestral archae, or the mitochondrion ancestor, or another yet unrecognized fusion partner). The fusion of two cells is an abrupt event that does not allow for the progressive evolution of genes and metabolism, and the hybrid resulting from such a fusion would thus have suddenly harbored sterols and sphingolipids within a single membrane system. As we have seen above, the formation of membrane domains in bacteria involves transitions between solid and fluid states, and there is no reason to suspect that this would have been any different in the bacterial ancestor(s) which did not contain sterols, and whose fusion to a sterol-containing one gave rise to the first eukaryotes. Some of the membrane proteins from this bacterial ancestor would thus have been adapted to function specifically when membranes were in a solid state. One of the advantages of bringing sterols and sphingolipids together in the same membranes must have been that the membranes became less susceptible to all-or-nothing switches between solid and fluid phases, in particular as a consequence of variations in temperature. If the co-habitation of sterols and sphingolipids had resulted in a complete inhibition of the formation of solid domains, however, many proteins that were previously activated by the formation of a solid microdomain in the bacterial membrane would have suddenly found themselves incapable of carrying out their functions, and it is hard to imagine that the cells resulting from the fusion would have survived such a sudden functional loss. Thus, if the solid domains were present in the bacterial ancestors, they almost certainly continued to form in the earliest eukaryotic ancestors. We perceive that what the co-existence of sterols and sphingolipids provoked in the earliest eukaryotes was not only a better resistance to sudden changes in temperature, but even more importantly, that it allowed early eukaryotes to depart from dichotomic responses to embrace the possibility of forming a larger diversity of membrane microdomains. In turn, this would open the doors to the formation of different types of domains, which could be linked to different intracellular responses, and pave the way to more elaborate developmental programs indispensable for the formation of multi-cellular organisms. A major difference between plants, fungi and animals is in the nature of their sterols, which may be related to the formation of different types of microdomains, leading to very diverse cellular processes, including the different mobility of their respective cells.

In the following section, we will present the existing evidence that such crystalline microdomains (in other words, gel or solid microdomains) very probably form in the membranes of the most evolved eukaryotes of today.

EVIDENCE FOR THE EXISTENCE OF SOLID DOMAINS IN EUKARYOTIC MEMBRANES

Since the discovery of Lo phases, gel or solid ordered domains have been almost universally considered as non-physiological and incompatible with a functional membrane in eukaryotes. To date, however, an increasing amount of evidence supports the existence of such domains in a whole variety of live cells, under physiological conditions, even if they continue to be referred to as exceptional situations (Jouhet, 2013).

In line with a hypothesis formulated by one of us some 10 years ago (Joly, 2004), we perceive that, among the whole diversity of different types of membrane microdomains, many may actually form through a process of crystallization around docks that would be mostly seeded by proteins. For example, it was shown at least in two cases that localized membrane rigidification can represent important defense mechanisms either as an initial signal following a temperature decrease in plants and other poikilotherms (Vaultier et al., 2006), or as a defense against antimicrobial peptides in yeast (Veerman et al., 2007). Whilst a membrane completely in Lo phase would be relatively insensitive to such signals, in a membrane harboring the cohabitation of Lo and Ld domains, the latter would turn into Lo domains (and/or become more rigid). This type of effect, however, would have a strong tendency to extend to the PM of the entire cell and therefore be too drastic. The formation of

small seeds of solid domains, still surrounded by membranes in Lo phase, would seem a more easily tunable mechanism for providing detectable yet controllable signals.

In yeast, FRAP experiments have demonstrated that there are PM proteins that diffuse very slowly, at speeds more compatible with gel than fluid domains (Valdez-Taubas and Pelham, 2003; Ganguly et al., 2009). In addition, single particle tracking techniques have demonstrated a complex confinement network and restricted diffusion of both lipids and proteins in animal cells. A commonly offered explanation for such behaviors is that certain proteins, linked to the cytoskeleton, are organized as corrals and pickets fences, and can hinder the diffusion of individual molecules (Kusumi et al., 2012). The architecture of such barriers, however, still remains to be fully elucidated, as well as the actual mechanism whereby proteins interacting with the cytoskeleton, i.e., in the aqueous phase of the cytoplasm, could hinder the diffusion of so many proteins and lipids diffusing in the plan of the membrane. As an alternative, we propose that some of the obstacles limiting the molecules' diffusion could correspond to areas in a gel state (**Figures 3E,F**). Accordingly, when molecules do get transiently trapped into gel nanodomains, it is the diffusion of those nanodomains which will be limited, quite possibly by other adjacent domains, or when the solid nanodomain is anchored to the cytoskeleton by one, or just a few, of its components. Such a mechanism would suffice to explain why the cytoskeleton has been found to play a pivotal role in the hindered diffusion of proteins and lipids in a multitude of different experimental systems (Kusumi et al., 2012), and does not call for the membrane to lie on a skeleton of actin filaments in such an intimate fashion that it can limit the diffusion of many of the membrane components, including certain lipids such as sphingolipids that are mostly found on the extra-cellular leaflet. It is worthy of note that the idea that the existence of solid domains could be limiting the diffusion of membrane components had already been clearly put forward by Nicolson almost 40 years ago (Nicolson, 1976). Moreover, in the model proposed by Kusumi et al. (2012), the existence of micrometer sized corrals defined by cytoskeleton and transmembrane proteins does not preclude the existence of nanometer sized lipid domains within each of those corrals. Of note, it has been proposed that in the case of cell-walled organisms, such as plants and yeast, the cell-wall plays a role similar to the cytoskeleton in hindering diffusion of PM components, as

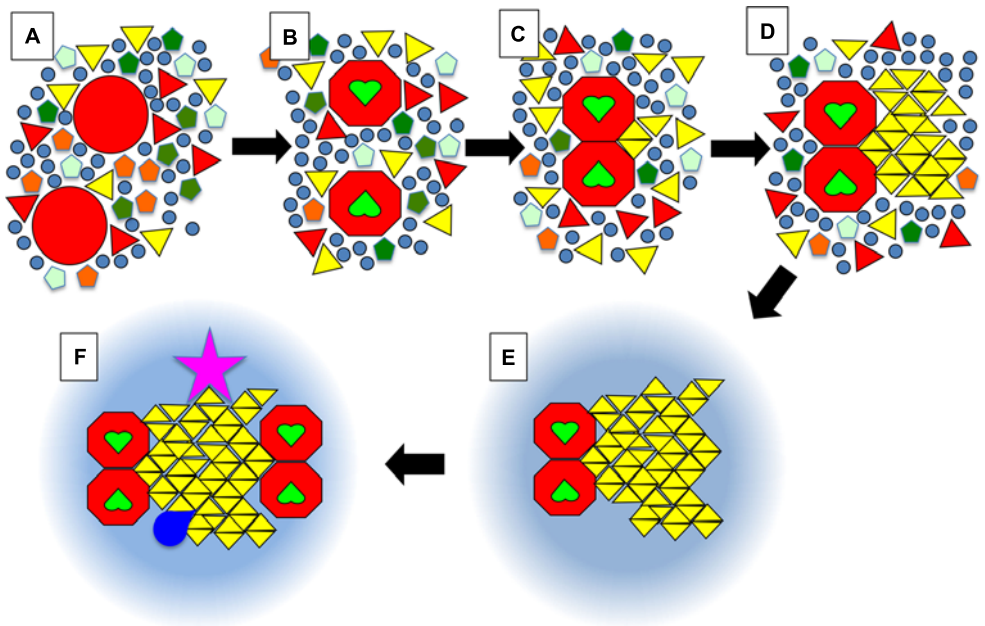

FIGURE 3 | Schematic view of the dock model. (A) Membrane proteins in a resting state (large red circles) are surrounded by a whole diversity of lipids such as phospholipids (pentagons), (glyco)sphingolipids (triangles), and sterols (circles). Some of these lipids would have a tendency to form solid phases at physiological temperatures but in the absence of stimuli the solid nanodomains do not reach a critical size that leads to further growth into a crystalline-like structure. The symbols can also be viewed as complexes formed by a small number of lipid molecules. **(B)** Upon activation, for example when receptors bind their ligand (green heart shapes), certain proteins will undergo a conformational change. **(C)** This will favor their homo- or hetero-dimerisation. The juxtaposition of proteins will create a new environment that will promote the binding of certain lipids such as one particular type of sphingolipid (yellow triangles). **(D)** Those bound lipids will act as the seed for the crystalline growth of a membrane dock, with the crystalline mesh dictating the specificity for one (or quite probably several) type(s) of lipid(s) with particular head groups and acyl chains, e.g., certain gangliosides (in the outer leaflet), phosphatidilinositol phosphates (PIPs) or even phosphatidic acid (PA) (in the inner leaflet). **(E)** After a crystalline dock forms, it will be surrounded by a ring of lipids with intermediate properties between So and Lo, which will most likely be quite similar to what is usually defined as rafts, i.e., enriched in sphingolipids and cholesterol. **(F)** Via percolation and enlargement of the catchment area, the rings could favor the exclusion or recruitment of other activated receptors, or of cofactors (kinases, GTPases …; dark blue shape). Importantly, some components of the dock (pink star) might be anchored to the cytoskeleton on the cytosolic side, or on the exoplasmic side to the extra-cellular matrix in mammalian organisms, or to the cell wall in plants and fungi.

reviewed in another paper of this issue (Martiniere and Runions, 2013).

Gel/fluid transitions at physiological temperature have actually been detected in certain cell types, such as sperm cells in latent state (Wolf, 1995). In mammalian cells, a common feature of many stress responses is the rise in ceramide levels, which is likely to promote local fluid >solid transitions and/or the formation of ceramide-rich platforms (Zhang et al., 2009; Stancevic and Kolesnick, 2010).

Using atomic force microscopy to observe supported ternary lipid bilayers, Giocondi et al. (2004) could directly follow the transitions upon increasing cholesterol content by treating the bilayer with cyclodextrin loaded with cholesterol. For the 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/bovine brain SM/cholesterol mix, increasing cholesterol resulted in the system going from gel/fluid to Lo/Ld to Lo (i.e., no domains). However, for the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/bovine brain SM/cholesterol mixture, they clearly observed a gel/Lo rather than a Ld/Lo intermediate situation and suggested this to be a plausible mechanism for domain formation in biological membranes, since those contain 1-saturated-2-unsaturated phospholipids rather than 1,2-diunsaturated ones. Until recently, the coexistence of Lo phase with So in binary phospholipid/sterol mixtures had not been observed by any imaging technique. However, this situation has changed and Lo/So nanodomains have now been observed directly by high-resolution atomic force microscopy in mixtures of DPPC/ergosterol (Vanegas et al., 2010).

Direct evidence for the existence of solid domains in the PM of a eukaryote was obtained by one of us with the budding yeast *S. cerevisiae* (Aresta-Branco et al., 2011). Using *trans*-parinaric acid, a fluorescent probe that has very different lifetimes in liquid and solid environments, it was demonstrated that, in cell cultures in mid-exponential phase, the sphingolipid-enriched domains are in a So state. Results of experiments performed with spheroplasts and a GPI-anchor remodeling mutant suggested that those domains might be points of interaction with the cell wall, possibly through GPI-anchored proteins. In addition, multiple evidence suggested that those solid domains were mostly located in the PM; they were strongly enriched in the PM fraction obtained by discontinuous gradient ultra-centrifugation, and in liposomes prepared with lipids extracted from purified PM as compared to intact cells and total lipids. A very recent report, performed by a completely independent group, has not only confirmed the formation of gel-like domains in the membranes of *S. cerevisiae*, but further documented the importance of sphingolipids in the process (Vecer et al., 2013).

At this stage, we feel that it is important to underline that the definition of a solid state in the context of biomembrane research does not equate with a frozen structure that would be completely static and rigid since such a structure would almost inevitably lead to cell death. The difficulty in using the terms of solid, liquid and gaseous is that those words are actually much better suited for describing macroscopic states. Membrane arrangements are, however, inherently micro-heterogeneous systems and highly anisotropic. Moreover, membrane microdomains are clearly micro- or even nanoscopic structures, involving only few hundreds of molecules, thus limiting the application of long-range/short-range order criteria for the distinction of phases.

Thus, when one considers things at the molecular level, time scales become a much more practical criterion to discriminate between those various states, in particular the average duration of interactions between neighboring molecules. In short, at the molecular level, one can say that in a gas molecules are separated from their neighbors most of the time, which they only encounter occasionally through collisions. In a liquid, molecules are in constant contact with their direct neighbors, but the interactions are too short for them to fully adapt their molecular structures to one another. In other words, in a liquid all intermolecular interactions last, on average, less than a microsecond. As soon as molecules start interacting with their neighbors for periods that are longer than a few microseconds, molecules will have enough time to modify their molecular architecture as a result of those interactions, and systems will start behaving more as solids than liquids (Elson et al., 2010). Under gel/solid conditions in a lipid bilayer, however, molecules still remain highly dynamic, and very significant diffusion does still occur, albeit much more slowly than in a liquid state. For example, even at 10^{-2} $\mu m^2/s$, the typical slow lateral diffusion rate of DPPC in a gel state, it takes only about one second for any two molecules randomly distributed in a 100 nm diameter LUV to encounter one another (this was calculated using the model of Tachyia, 1987; de Almeida et al., 2002).

THE SOLID DOCKS HYPOTHESIS

In the following paragraphs we propose a model where the formation of many of the microdomains that appear in the PM of eukaryotes involves components switching from a fluid or amorphous state to a crystal-like state, based on the specific recruitment of particular lipids and proteins into solid nanodomains.

From a thermodynamic point of view, the recruitment of lipids into a solid crystalline-like domain will be energetically favorable for those specific lipids that acquire a solid type conformation due to favorable molecular interactions with the nucleus, i.e., the solid ordered nanodomains. This "crystalline recruitment" will be specific, therefore different lipids will be recruited into different types of domains. Crystal growth is not governed solely by thermodynamics, as it requires individual molecules to diffuse and to encounter the nucleation seeds, and/or the diffusion of the nanodomains toward one another. Solid nanoclusters will grow and decay until a critical size is reached and the crystallization process continues to form a large and stable crystalline domain or platform. Hence, most of the times, the solid clusters are highly labile entities that form and collapse, which actually corresponds to features that have been attributed to lipid rafts, and not at all frozen structures as they are usually described. Furthermore, the reverse switch from a domain back to the liquid phase will tend to be much less probable, resulting in much longer times of residence inside solid than inside Lo domains. This type of specific recruitment into solid domains would thus provide a possible explanation for the parallel formation of different types of microdomains at the surface of a single cell, and over timescales that would be much more compatible with those necessary for cellular events such as intracellular transport or the assembly of signaling platforms, which usually occur over periods

going from seconds to minutes or even hours (Papin et al., 2005; Hoffman et al., 2011). Incidentally, recent advances in deciphering the general process of crystallization of solutes suggest that it may often involve the initial formation of nanoparticles which subsequently join to form a larger crystal (Teng, 2013). Crystalline membrane nanodomains could thus correspond to such nanoparticles, but their growth would be limited by availability of additional components, and by cellular responses such as the constant turnover due to an intense trafficking inside the cells.

Within such a model, where crystalline structures are more stable than the fluid ones, their disruption would not simply happen by removing the stimulus that initially led to their formation. It would thus be necessary to remove the solid platform from the membrane, e.g., by endocytosis, and then follow membrane recycling processes. This is in fact known to occur for most surface receptors that form dimers or oligomers upon stimulation. Alternatively, changes in the physical environment following stimulation, such as a change in the lipid composition, in the membrane electric dipole potential, or in ionic potential could lead to a novel situation where the solid state would no longer be more stable than the fluid one. For example, it is well established that several signaling transduction pathways involve a variety of mechanisms such as activation of lipases, alteration of lipid biosynthetic rates, strong alterations in ionic gradients, and most notably in the levels of cytosolic calcium, or even the alteration of membrane binding affinity of highly cationic proteins at the cytosolic side of the PM. All these events have been recognized for some time as important control mechanisms of the fluctuations in the physical state and composition of dynamic membrane domains (Heimburg, 2003). They could in fact function as negative feedback loops, whereby a signaling platform would activate a series of cellular events which would in turn lead to the "melting" of the signaling platform.

Another important aspect of the formation of such domains concerns the homogenizing role of sterols. As was extensively developed earlier, sterols tend to act as homogenizers, by preventing the occurrence of an all-or-nothing liquid to solid transition, and by promoting the co-existence of lipids with very different T_m's into an intermediate state between Ld and So. In some cases, sterols may become incorporated into the crystalline lattice, but in most cases, given their capacity to disrupt solid phases, they would presumably have to be excluded from solid structures. Quite possibly, however, sterols could in certain instances accumulate in one leaflet to fill the gaps, for example if the crystalline lattice only involves one leaflet of the bilayer, or if that lattice involves lipids with very long fatty acid chains that can span into the opposite leaflet.

In **Figure 3**, we present an ultra-schematic representation of the dock model, where the dimerisation of a membrane receptor upon binding of its ligand provides the seed for the formation of a crystalline dock comprised of a single lipid species or lipid complex (**Figures 3A–D**). As depicted in the lower part of **Figure 3** (i.e., sequence **E,F**), rings would be expected to form around the crystalline docks, corresponding to an area of transition in order, thickness and other physical properties between the solid center and the more fluid general membrane environment. Such rings could even have a role to play in the enrichment of certain proteins, or of particular lipid species, for their recruitment into the central docks. In this regard, such rings would be quite akin to the lipid shells proposed by Anderson and Jacobson (2002). Such arrangements would not represent a significant entropic cost because they would have properties in between the solid platforms and the remainder of the membrane, avoiding for example a large hydrophobic mismatch. This organization of domains was actually observed in DOPC/DPPC/cholesterol GUVs (**Figure 2A**) and was also predicted, by FRET experiments, to be the one that occurs when highly ordered ceramide/sphingomyelin-enriched gel domains form within PC/SM/cholesterol membranes with Ld/Lo coexistence (Silva et al., 2007).

Earlier we alluded to recent evidence that the PM of eukaryotic cells could be mostly in a Lo phase. Since, by nature, living organisms are not in thermodynamic equilibrium, one possible way of describing the lateral organization of membranes is to consider that there are membrane regions behaving as an amorphous state. Provided that the system is near or above the so-called glass transition temperature T_g, amorphous states can still show high levels of molecular diffusion (Graeser et al., 2010). Therefore, it could turn out that the Lo-like state of the PM of live cells actually corresponds to an amorphous state with high lateral diffusion.

Within the framework of an out-of-equilibrium description of the cell membrane, the density fluctuations characteristic of amorphous states could explain membrane properties that are usually ascribed to the Ld/Lo coexistence paradigm. For example, it has recently been established that, under particular thermodynamic conditions, a two-dimensional fluid can become a stable mosaic of small differently ordered clusters which can be described as a state of micro-phase separation between amorphous and crystalline domains (Patashinski et al., 2013).

An amorphous state is not an equilibrium state and is thus of high Gibbs energy (aka free energy or free enthalpy). Consequently, it may present different degrees of short range order and rigidity. The formation of the crystalline domains would entail the use of that high energy, via a transition from amorphous to crystalline state. The discussion in the previous paragraphs about nucleation and microdomain formation does, however, remain valid because the mechanism for crystal growth is the same as for a liquid-to-crystalline transition (Hancock and Zografi, 1997). Furthermore, another characteristic of the amorphous state is the presence of density fluctuations (Wiegand, 1979), and composition fluctuations assigned to critical behavior were observed in membrane model systems of lipid rafts (i.e., with liquid-liquid phase separation) and also in giant PM vesicles (Honerkamp-Smith et al., 2009).

As underlined by Quinn (2010), micro-phase separation can also be observed in model bilayers comprised of ternary mixtures of asymmetric sphingolipids, glycerolipids and cholesterol. Their examination by a number of biophysical methods has led to a set of results that could be interpreted as formation of binary complexes of asymmetric sphingolipids and glycerophospholipids that exclude cholesterol, and which have diffusional characteristics more akin to a gel than a Lo phase (Filippov et al., 2006). In the past, the group of Quinn has characterized quasi-crystalline phases in co-dispersions of phosphatidylethanolamine

and glucocerebroside (Feng et al., 2004). More recently, Quinn (2010) has proposed a "lipid matrix model" for the formation of rafts, whereby the seeding of stoichiometric complexes between certain sphingolipid and phospholipid species with a highly ordered or quasi-crystalline organization would result in solid-like phases in biological membranes under physiological conditions. According to this model, asymmetric sphingolipids with very long chain fatty acids (e.g., C24), even if only present in small amounts in cell membranes, are responsible for the formation of quasi-crystalline phases which exclude cholesterol; the glycosyl component of those sphingolipids would provide additional selectivity through interaction with proteins. This type of model for the co-existence of different types of solid domains is supported by extensive experimental data obtained with model membranes made of binary lipid mixtures which showed solid-solid immiscibility even when the lipids differed only in their headgroup or in the acyl chain length (Mabrey and Sturtevant, 1976; Silvius and Gagne, 1984; Graham et al., 1985; Marsh, 2013).

In fine, the "Lipid matrix model" proposed by Quinn (2010) is effectively very similar to the model of solid docks (Joly, 2004). In both cases, the basic idea is that different solid-phase seeds would exist, mostly due to proteins inducing different crystalline lattices, thus leading to the formation of different domains that would specifically recruit certain solid-forming lipids. Since such lipids only comprise a small percentage of the membrane lipids, the growth of those domains would be self-limiting, but would be expected to suffice for the recruitment of additional proteins, either identical to or different from those having seeded the dock. Good candidates for proteins that could promote and/or stabilize such domains are surface receptors that tend to homo- or hetero-dimerize (or even multimerize) upon stimulation (as stated above, stable crystal growth depends on the attainment of a minimum critical size of the nucleation center), as well as components anchored to cell-wall proteins in fungi and plants, and cytoskeleton proteins in all eukaryotes. Such components would have a major influence on the movements of all the dock's components, and consequently also on the diffusion of the free molecules in the direct vicinity of the dock, which would explain why disrupting the cytoskeleton can have such a dramatic effect on the existence of PM microdomains and/or on the diffusion of proteins and lipids that do not directly interact with cytoskeleton components.

TECHNICAL CHALLENGES

To date, only a handful of studies have documented, or even just suggested the existence of solid domains in eukaryotic membranes. This could be taken to imply that such solid domains only occur exceptionally, but we suspect that this may actually be due more to the fact that people have not looked for them, which is in large part related to the almost complete absence of clear-cut and/or foolproof methods for the detection of such solid nanodomains in living cells under physiological conditions. Furthermore, and as underlined earlier, if one considers the amount of sterols in the PM of all eukaryotic cells, comparison with lipid phase diagrams established in membrane model systems leads to the prediction that most of the PM will be in a Lo state, in agreement with recent results obtained in cells. Other types of domains would thus be a minority, and therefore difficult to detect. We do, however, remain convinced, and hopeful, that people will start to find evidence for the existence of solid domains in the PM of eukaryotic cells when they start looking for them, with the proper tools. In this respect, Heimburg (2003) has pointed out that the absence of a pronounced melting peak in many biological systems cannot be directly interpreted as an absence of melting events, since the chain melting may be spread out over a large temperature range due to the large variety of lipid components, rendering it difficult to detect such transitions.

One of the main hurdles for the characterization of microdomains in live cells has been attributed to their small size of a few tenths of nanometers, and thus below the diffraction limit of visible light, as well as their very dynamic nature, which have prevented their direct observation by standard light microscopy. Over the past few years, a score of technological developments have, however, been pushing back the limits of this resolution well below 100 nm, and a review focused on the advances of super-resolution microscopy relevant to understanding membrane lipid domains can be found in the same issue as our paper (Owen and Gaus, 2013). For example, using STED-FCS to reach resolutions of the order of 30 nm, Mueller et al. (2011) documented anomalous diffusion of sphingolipid probes at the surface of live cells, leading them to suggest the existence of domains involving strong intermolecular interactions either between lipids, or between lipids and proteins.

To date, a very large proportion of studies that have been carried out on live cells have relied on approaches that were not suited to discriminate between Lo and So phases. For example, fluorescent probes such as Laurdan or di-4-ANEPPDHQ have been extensively used to study membrane order, but those probes do not allow to fully discriminate between either Lo and So or So and Ld phases. To document the existence of solid domains in a variety of cells, comparison of anisotropy values with probes such as DPH could be a more hopeful approach, but remains limited in its capacity to detect nanoscopic solid domains, among other disadvantages such as UV excitation wavelengths or photoinstability. Approaches based on the fluorescence lifetimes of certain fluorescence probes can lead to the definitive detection of solid phases, as was achieved in yeast with tPNA (Aresta-Branco et al., 2011; Vecer et al., 2013). The very high sensitivity of this probe to photo-degradation does, however, preclude its use for microscopy. None of the above probes are thus ideal to discriminate between So and Lo domains in single cells, and new probes that are photostable and easy to incorporate into cells are still sorely needed. In this regard, the combination of molecular rotor probes and FLIM imaging seems a very hopeful approach since it was used to document that the microviscosity of the inner membrane of *B. subtilis* dormant spores was very high, suggesting that it was in a gel state. Interestingly, this viscosity decreased significantly upon germination (Loison et al., 2013). One of the main difficulties in using fluorescent probes to investigate the existence of crystalline domains, however, is that, if there is a specificity of recruitment into crystalline nanodomains, many probes will most likely be excluded from such domains.

NMR spectroscopy provides a definite way to discriminate between Lo and So states, but the studies that have used FTIR

and NMR spectra to study membrane order in mammalian cells have almost exclusively relied on the incorporation of deuterated cholesterol derivatives, which one would expect to be excluded from solid domains. Notwithstanding the fact that solid nanodomains are only ever expected to represent a minute portion of a cell's PM, and that NMR-based studies do not distinguish into which lipid species the isotopic labeled component was integrated, it would still be interesting to carry out similar experiments with deuterated lipid precursor(s) that would incorporate preferentially into solid-forming lipids such as sphingolipids with long chain fatty acids.

Another approach that may be used to assess whether or not solid lipid phases are present in signaling platforms may be derived from that described by Harder and Kuhn (2000) in which they incubated lymphocytes with magnetic beads coated with anti-T-cell receptor antibodies and fragmented the membranes by cavitation before isolating the membranes containing the signaling complexes surviving the mechanical disruption process. As an alternative, we have also used exosomes as a source of naturally isolated microdomains, and found them to be in a very ordered state by DPH anisotropy measurements (Carayon et al., 2011), as well as Laurdan spectroscopy and tPNA lifetime measurements (unpublished data). Chemical mapping after metabolic labeling, such as developed by the group of Mary Kraft is yet another hopeful approach (Kraft, 2013), although this only gives information on the concentration enrichment of a given lipid species, but does not indicate which kind of phase the lipids are in.

With the tools and methodological approaches available today, the study of membrane lipid domains in physiological conditions thus remains a difficult problem to tackle and the design of clear-cut experiments that will allow the unquestionable discrimination between Lo and So phases will first require that scientists start looking for solid domains, but will also probably have to await the development of new tools, and/or new approaches.

CONCLUSION

From the perspective of the fluid mosaic model, the formation of gel/So/crystalline domains in biological membranes was initially perceived by many as very unlikely, but experimental evidence keeps accumulating suggesting their possible existence. Furthermore, the formation of such crystalline nanodomains could confer the stability required for the kind of time frames and selectivity for cellular events to unfold, such as intercellular transport or assembly of signaling platforms.

AUTHOR CONTRIBUTIONS

Rodrigo F. M. de Almeida and Etienne Joly contributed equally to the ideas developed in this manuscript, and to its writing.

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Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing?

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The structure, dynamics, and stability of lipid bilayers are controlled by thermodynamic forces, leading to overall tensionless membranes with a distinct lateral organization and a conspicuous lateral pressure profile. Bilayers are also subject to built-in curvature-stress instabilities that may be released locally or globally in terms of morphological changes leading to the formation of non-lamellar and curved structures. A key controller of the bilayer's propensity to form curved structures is the average molecular shape of the different lipid molecules. Via the curvature stress, molecular shape mediates a coupling to membrane-protein function and provides a set of physical mechanisms for formation of lipid domains and laterally differentiated regions in the plane of the membrane. Unfortunately, these relevant physical features of membranes are often ignored in the most popular models for biological membranes. Results from a number of experimental and theoretical studies emphasize the significance of these fundamental physical properties and call for a refinement of the fluid mosaic model (and the accompanying raft hypothesis).

Keywords: raft hypothesis, fluid mosaic model, membrane lateral pressure profile, membrane compositional fluctuations, membrane curvature, membrane domains, membrane lateral organization

BRIEF HISTORICAL OVERVIEW

Current views on structural and dynamical aspects of biological membranes have been profoundly influenced and to some extent biased by the fluid mosaic model, proposed by Singer and Nicolson (1972). This model supports the idea of lipids forming a more or less randomly organized fluid, flat, bi-dimensional matrix in which proteins perform their distinct functions. Although lipid-mediated lateral heterogeneity in membranes was concurrently described during the 1970s, this feature was not considered in the nascent Singer and Nicolson model.

Early proof that lipids could laterally segregate forming physically distinct "domains" in model membrane systems was reported in the 1970s (Phillips et al., 1970; Shimshick and McConnell, 1973; Grant et al., 1974; Lentz et al., 1976; Schmidt et al., 1977). Along with these observations, it was proposed that lipid compositional heterogeneity may play a role in the modulation of relevant physical properties of natural membranes. Lipid lateral segregation, which might arise under particular environmental plausibly found in physiological states, would be one of these (Gebhardt et al., 1977; Schmidt et al., 1977). Furthermore, membrane regions induced by lipid-protein interactions were proposed as a physical basis for membrane-mediated processes (Marcelja, 1976; Mouritsen and Bloom, 1984; Sackmann, 1984). These and other questions and theoretical possibilities were addressed by various researchers on several occasions (Träuble, 1976; Op den Kamp, 1979; Thompson and Tillack, 1985; Vaz and Almeida, 1993).

To account for lipid-mediated lateral heterogeneity alternative models of biological membranes have been proposed. For example, the "plate model," introduced by Jain and White (1977), proposed that separation of ordered regions from disordered (fluid) regions occurs in biological membranes as a natural consequence of specific intermolecular interactions and lattice deformation. At around that time, Israelachvili proposed another model to account for the need of membrane proteins and lipids to adjust to each other (Israelachvili, 1977). This insight provided the conceptual framework for "the mattress model" proposed by Mouritsen and Bloom (1984) which suggests that, in membranes, lipids, and proteins exhibit interactions associated with a positive Gibbs energy caused by a hydrophobic matching condition that can lead to elastic distortions of the membrane matrix. This type of phenomenon in turns gives rise to interfacial tension between lipid and proteins, resulting in clustering of specific lipid molecules around a protein or lipid-mediated protein–protein interactions (due to capillary forces). In addition, a model accounting for the importance of the cytoskeleton and the glycocalyx on membrane organization was developed by Sackmann (1995)[1]. Regrettably, many of the important physical mechanism highlighted by these models are generally ignored when membrane-related phenomena are

[1]Space limitations imposed by the journal for a "minireview article" prevent us to discuss in detail the possibility that cortical actin and its associated proteins could compartmentalize the plasma membrane into distinct domains. Notice however that other contribution in this special issue deals with this topic.

addressed (e.g., transport processes, action of second messengers), and the general outlook introduced by the fluid mosaic model still prevails (Bagatolli et al., 2010; Bagatolli, 2012).

A proposal regarding the role of lipid heterogeneity came along with the "raft hypothesis," which has its origin in observations reported by Simons and van Meer (1988). These authors envisaged the formation of lipid domains as an early event in the sorting process in the plasma membrane of epithelial cells. This hypothesis was subsequently generalized, proposing the existence of microdomains ("rafts") enriched in sphingolipids and cholesterol. These domains were surmised to be functionally associated with specific proteins involved in intracellular lipid traffic and cell signaling (Simons and Ikonen, 1997). The idea that these rafts, by being enriched in cholesterol, should have special physical properties arose from original observations in model membranes reported by Ipsen et al. (1987), showing that under particular conditions cholesterol generates the coexistence of liquid-disordered (l_d) and liquid-ordered (l_o) lamellar phases. The liquid-ordered phase combines free rotational and translational diffusion of lipids (as found in the L_d phase) with a low proportion of *gauche* rotamers in the hydrocarbon chains (i.e., high order rather than low order), as is usually found in the solid ordered (s_o, or gel) phase (Ipsen et al., 1987). Since 1997, the raft hypothesis has become very popular among researchers in the biosciences, spawning thousands of projects and publications in multiple areas of cell biology, biochemistry, and biophysics. However, accurate definitions of the physical phenomena that would underlie the raft hypothesis are still lacking, a fact that has resulted in numerous reformulations over the last few years. One of the latest definitions states that rafts are "...fluctuating nano-scale assemblies of sphingolipid, cholesterol, and proteins that can be stabilized to coalesce, forming platforms that function in membrane signaling and trafficking" (Lingwood and Simons, 2010). In this definition, "rafts" are claimed to exist in an "ordered phase" (defined as a "raft phase") that "...is not similar to the liquid-ordered phase observed in model membrane systems." The term *phase* (appropriated from systems at thermodynamic equilibrium) is used in the context of cellular membranes, somehow overlooking that local equilibrium conditions need to hold first. It remains to be established whether membranes are best described as being near local equilibrium at some time scale (thus allowing *phase separation*), or whether they can be more appropriately perceived as metastable regions caused by fluctuations originating from non-equilibrium conditions. Perhaps one of the more questionable aspects of the raft hypothesis was its original operational definition, which was based on detergent extraction methods. Using detergent-extraction techniques is influenced by the way protein chemists work, isolating specific membrane proteins from biological material. However, membranes are self-assembled macromolecular structures in which a range of different molecular species organizes due to weak physical and thermally renormalized forces. Seen from this point of view, adding detergents to membranes is the last thing you would do to study lateral organization. Even though it has been shown that detergents impinges a completely different structural and dynamical features to membranes (Heerklotz, 2002; Sot et al., 2006), the identification of rafts based on various detergent extraction methods is still loosely accepted today. At this stage, however, the fact that detergents do not isolate preexisting membrane domains is more widely recognized (Lingwood and Simons, 2010). Last but not least, conclusive experimental evidence about the existence of rafts in the plasma membrane remains elusive.

RELEVANT PHYSICAL PROPERTIES OF MEMBRANES

The structure, dynamics, and stability of lipid bilayers are controlled by thermodynamic forces, leading to overall tensionless membranes with a distinct lateral organization and a conspicuous lateral pressure profile (reviewed in Bagatolli et al., 2010; Mouritsen, 2011a,b). The transverse structure is a noticeable feature of a lipid bilayer, and is far from that of an isotropic fluid slab of hydrocarbons. Bilayers display a distinct lateral stress- or pressure profile (Brown, 1994; Cantor, 1997, 1999a,b; Marsh, 2007) as illustrated in **Figure 1A**. The physics behind this profile is based on simple mechanics. In mechanical equilibrium in the tensionless state, the integral of the difference between the normal pressure and the lateral pressure, $p_N(z)$–$p_L(z)$, has to become zero. However, the variation of the lateral pressure across the 5 nm thick membrane goes from positive, expansive pressures in the head group region, over regions of negative, tensile pressures in the interfacial regions, to expansive, positive pressures in the acyl-chain region, as illustrated in **Figure 1A**. These variations can easily amount to the equivalent of hundreds of atmospheres pressure. It is this very stressful environment integral membrane proteins have to come to terms with. The lateral pressure profile has recently been computed in 3D (in contract to the initial 1D) and used to determine the effect of the 3D transmembrane pressure distribution on membrane protein activation (Samuli Ollila et al., 2011).

Bilayers are also subject to built-in curvature-stress instabilities that can be locally or globally released in terms of morphological changes (Mouritsen, 2011a,b, 2013). A crucial regulator of the bilayer propensity for forming curved structures is the lipid average molecular shape. It is possible to describe lipid phase behavior via a simple geometric property of the lipid molecule, the so-called Israelachvili–Mitchell–Ninham packing parameter, $P = v/al$, where v is the molecular volume, a is the cross-sectional area of the head group, and l is the length of the molecule (Israelachvili, 1992), cf. **Figure 1B**. Of course a lipid molecule in a dynamic lipid aggregate cannot be assigned a shape as such, and the geometric parameters v, a, and l should therefore be considered as average molecular properties. Still, the value of P turns out to be surprisingly useful in predicting the structure of a lipid aggregate. For instance, if the lipid composition in the two leaflets of a thermodynamically stable bilayer changes (e.g., upon lipase action or incorporation of other lipids), and these new lipids have values of P different from unity, the bilayer (via the lateral pressure profile) will suffer from a built-in curvature stress. Such monolayers would curve (**Figure 1C**) if they were allowed to do so and not being confined to constitute a stable bilayer, leading to the formation of non-lamellar and curved structures (**Figure 1B**). Via the curvature stress, molecular shape mediates also a coupling to membrane-protein function and provides a set of physical mechanisms for formation of lipid domains and laterally differentiated regions in the plane of the membrane (Mouritsen, 2013).

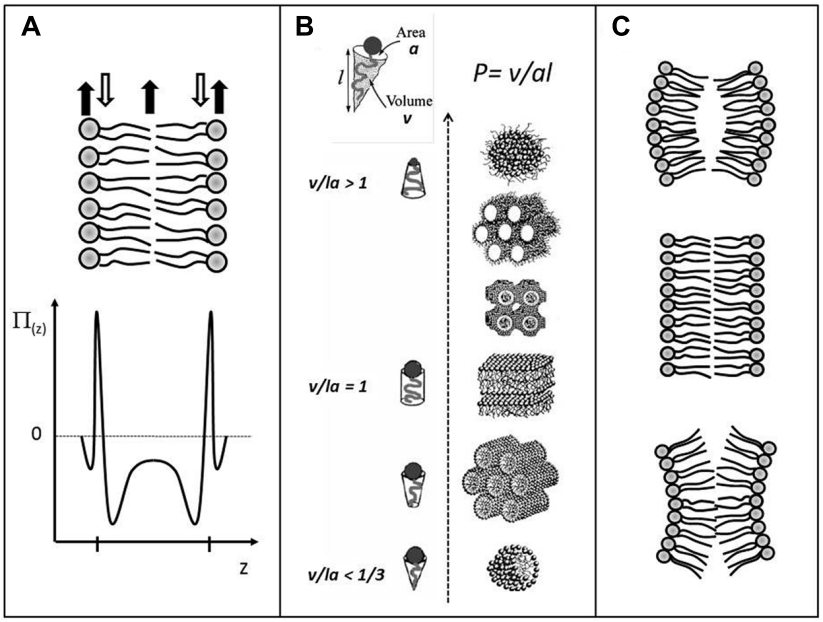

FIGURE 1 | Schematic illustrations of: (A) the lateral pressure profile, p(z), of a lipid bilayer, revealing regions of expansive (positive) pressures and regions of large tensile (negative) pressures; **(B)** lamellar and non-lamellar lipid aggregates formed by self-assembly processes in water. The different structures have different senses of curvature and are arranged in accordance with the value of the phenomenological molecular packing parameter *P*; **(C)** Lipid monolayers with positive, zero, and negative (from top to bottom) curvature determined by the shape of the lipid molecules. Stable lipid bilayer (center) formed by two opposing lipid monolayers. If the monolayers were not constrained by being in the bilayer, they may curve as shown at the top and the bottom illustrations. In the latest cases, the stable bilayer would suffer from a built-in curvature stress. Adapted from Mouritsen (2011a) with permission.

ARE THE "FLUID MOSAIC" AND THE ACCOMPANYING RAFT HYPOTHESIS THEN SUITABLE MODELS TO DESCRIBE FUNDAMENTAL FEATURES OF BIOLOGICAL MEMBRANES?

It has been suggested that the fluid mosaic model of membranes has been successful because it does not bias the researcher too strongly, allowing for broad interpretations of new experimental data and novel theoretical concepts (Mouritsen and Andersen, 1998; Bagatolli et al., 2010). This suggestion can somehow be extended to the raft hypothesis. For example, "rafts" have been variously referred to as *constitutive structural elements* of cellular membranes (disregarding important dynamical aspects of membranes), proposed to exist in almost all biological membranes (overlooking their rich compositional diversity), and structured in some sort of liquid-ordered phase (although studies demonstrating the occurrence of local equilibrium conditions are very scarce). Moreover, the assertion of a liquid-ordered structure is seldom verified directly but only indirectly by pointing to the high local concentration of cholesterol. In fact the determination of a liquid-ordered structure has turned out to be an elusive problem even in simple model membranes and only recently has some hard evidence been established in model membranes by combining scattering data and model simulations (Rheinstädter and Mouritsen, 2013). One of the most recent and credible studies of "rafts" in live cells combine fluorescence correlation spectroscopy (FCS) with stimulated emission depletion microscopy (STED; Eggeling et al., 2009). This study suggested the existence of cholesterol concentration dependent domains of sizes around 20 nm, where plasma membrane proteins dwell for periods of 10–20 ms. The models used for data analysis have, however, challenged because they rely on the assumption of locally "flat" surfaces (supported by the fluid mosaic model) and ignore the already documented complex topography of the plasma membrane (Adler et al., 2010). One way or another, it is clear that the raft hypothesis extends the mosaic nature of the membrane proposed by Singer and Nicolson to include now functionally important distinct *fluid* domains, selective in terms of *both* protein and lipid components. Notice that the generic view of the fluid mosaic model prevails again and no reference is made to relevant membrane physical features such as the transbilayer structure (and the associated lateral pressure profile; Cantor, 1997), curvature stress (Miao et al., 1994), instabilities toward non-lamellar symmetries (Seddon and Templer, 1995), coupling between internal membrane structure or hydrophobic matching (Mouritsen and Bloom, 1984), and intrinsic membrane permeability near phase transitions (Heimburg, 2010). Thus incorporation of other, more realistic, models, or modifications of the most popular ones are urgently required to interpret membrane related phenomena.

NEW CHALLENGES AND FUTURE PERSPECTIVES

Are there examples from naturally occurring membranes displaying micrometer-sized domains as observed in model membrane systems? Yes, in very specialized membranes such as lung surfactant and skin stratum corneum, where lipids are the principal components, membrane-cytoskeleton anchorage is lacking, and

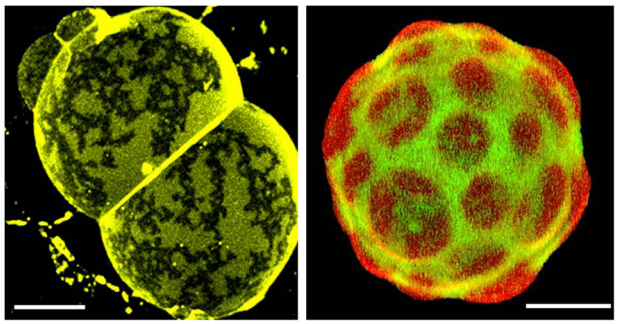

FIGURE 2 | Confocal fluorescence images of natural membranes showing micrometer-sized domains. Left: skin stratum corneum lipids membranes from human. This specialized membrane contains 11 different ceramides, cholesterol, and long chain (C_{24}–C_{26}) fatty acids in a ~1:0.9:0.4 mol ratio, and displays coexistence of two gel-like phases (Plasencia et al., 2007). The membrane is labeled with $DiIC_{18}$, $T = 32°C$ (which represent skin physiological temperature. Right: pulmonary surfactant membranes from pig. This specialized membrane is mainly composed of phospholipids and small amounts of specifically associated proteins (SP-B and SP-C). Among the phospholipids, significant amounts of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol are present, both of which are unusual species in most animal membranes. Mono-unsaturated phosphatidylcholines (PC), phosphatidylinositol, and neutral lipids including cholesterol are also present in varying proportions (Bernardino de la Serna et al., 2004). This natural membrane is labeled with $DiIC_{18}$ (red) and Bodipy-PC (green) and is displaying coexistence of lo and ld-like phases, $T = 37°C$. Scale bars are 10 μm.

local equilibrium conditions are likely attainable (Bernardino de la Serna et al., 2004; Plasencia et al., 2007), cf. **Figure 2**. Other examples have been reported, such as platelets upon activation (Gousset et al., 2002), macrophages (Gaus et al., 2003), T-cells (Klammt and Lillemeier, 2012), yeast (Spira et al., 2012), red blood cells (Sanchez et al., 2012; D'Auria et al., 2013), fibroblasts (Frisz et al., 2013) although it is not yet clear whether these observations are controlled by the same mechanisms. The message here is that generalizations can be perilous, and it is probably a good idea to pay attention to the compositional diversity of different membranes, including the way that processes evolve (local equilibrium vs. non equilibrium conditions).

Since conclusive experimental evidence about the existence of domains in live cell plasma membranes remains elusive, fluctuations observed at compositions near the critical point, reported from phase diagrams of ternary mixtures containing cholesterol (Veatch et al., 2007; Idema et al., 2009), have been considered as a potential physical basis to infer the presence of fluctuating nanoscale assemblies in plasma membranes (or rafts). This equilibrium phenomenon is claimed to be relevant to membrane function (Veatch et al., 2008). As mentioned previously (Bagatolli et al., 2010), critical-point phenomena are singular in nature and hence it is unlikely that they *per se* play a role in biological regulation. For example, minuscule mistuning near a critical point may lead dramatic changes in membrane structure and dynamics. It is more likely that a related phenomenon associated with non-equilibrium critical behavior, or self-organized critical behavior, which is robust and needs no tuning, may play a role in biology (Jensen, 1998). Understanding these kinds of processes will prove very challenging, particularly considering that the biophysics of membrane organization under non-equilibrium conditions is in its infancy (Sabra and Mouritsen, 1998; Girard et al., 2005; Turner et al., 2005; Foret and Sens, 2008; Fan et al., 2010; Bouvrais et al., 2012). In order to understand how membrane heterogeneity becomes controlled by the non-equilibrium state of the lipid matrix, it is vital to explore new experimental models and theory-based approaches (Bagatolli et al., 2010; Bagatolli, 2012). For example, *active membrane systems* subject to transport, signaling, and enzymatic processes should be experimentally designed and studied (Bouvrais et al., 2012). Last, but not least, it is worth mentioning that the behavior of biological systems (including membrane related processes) is generally viewed in terms of mass-action kinetics. However, natural systems exist far beyond the dilute concentration limit; consist of molecularly crowded environments with variable water activity and a collection of (small) sizes. The impact of these conditions on membrane structure and dynamics is still obscure and waiting to be elucidated.

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Toward atomic force microscopy and mass spectrometry to visualize and identify lipid rafts in plasmodesmata

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Plant cell-to-cell communication is mediated by nanopores called plasmodesmata (PDs) which are complex structures comprising plasma membrane (PM), highly packed endoplasmic reticulum and numerous membrane proteins. Although recent advances on proteomics have led to insights into mechanisms of transport, there is still an inadequate characterization of the lipidic composition of the PM where membrane proteins are inserted. It has been postulated that PDs could be formed by lipid rafts, however no structural evidence has shown to visualize and analyse their lipid components. In this perspective article, we discuss proposed experiments to characterize lipid rafts and proteins in the PDs. By using atomic force microscopy (AFM) and mass spectrometry (MS) of purified PD vesicles it is possible to determine the presence of lipid rafts, specific bound proteins and the lipidomic profile of the PD under physiological conditions and after changing transport permeability. In addition, MS can determine the stoichiometry of intact membrane proteins inserted in lipid rafts. This will give novel insights into the role of membrane proteins and lipid rafts on the PD structure.

Keywords: mass spectrometry (MS), atomic force microscopy (AFM), lipid raft, membrane proteins, plasmodesmata (PDs)

INTRODUCTION

Plamodesmata (PDs) are nanopores connecting the cytoplasm of adjacent cells to facilitate intercellular communication. PDs generate symplastic communication pathways to transport small molecules below the size exclusion limit and selected bigger molecules. This in turn plays important roles for the cell fate and development, viral movement and transport of metabolites and miRNA (Bouyer et al., 2008; Lucas et al., 2009; Carlsbecker et al., 2010; Miyashima et al., 2011; Furuta et al., 2012)

PDs are structurally composed by a continuous plasma membrane (PM) between two adjacent cells with an axial highly packed central element of endoplasmic reticulum named desmotubule (Hepler, 1982; Tilney et al., 1991; Ding et al., 1992). The cell wall surrounding the channel is rich in pectin and depositions of β-1,3-glucan (callose) in the plasmodesma (PD) neck zone. To date there is consensus that these depositions regulate the molecular size of the transported molecules through PD (Roy et al., 1997; Botha and Cross, 2000; Levy et al., 2007; Guseman et al., 2010). In recent years, there have been considerable efforts carried out to determine the molecular composition of the PD. In particular, using nano-LC ion trap MS/MS, Fernandez-Calvino et al. (2011) have done a proteomic analysis of PD vesicles and identified 1341 proteins that putatively belong to the PD (five of them were confirmed to be located inside the PD by confocal microscopy experiments), including glycosylphosphatidyl inositol (GPI)-anchored proteins. This family of proteins are anchored to the membrane and present high affinity for sterol containing lipid bilayers, which suggest that PD membrane could be forming lipid rafts (Mongrand et al., 2010; Salmon and Bayer, 2012). Membrane lipid rafts are defined as dynamical assemblies of sphingolipids and sterols (Lingwood and Simons, 2010). Supporting this hypothesis, Remorin protein, a lipid raft marker, was accumulated in the PD (Raffaele et al., 2009). Nevertheless, there is no experimental evidence showing the presence of lipid rafts in PDs, neither their lipid composition nor the location of intact specific membrane proteins. In this perspective article we propose a series of experimental approaches to get insights into these important issues by using atomic force microscopy (AFM) and mass spectrometry (MS).

ATOMIC FORCE MICROSCOPY IMAGING

AFM was invented (Binnig et al., 1986) to analyse only conductive samples and since then has advanced enormously specially with the design of new methodologies to study biological samples such as proteins, DNA and lipid bilayers under physiological conditions (Muller, 2008; Shahin and Barrera, 2008; Picas et al., 2012; Whited and Park, 2013). Basically AFM consists of a sharp tip around 10 nm radius attached to a flexible cantilever scanning over a sample to reconstruct its three dimensional topography. Because of the tip width further geometric deconvolution is needed to improve the sample lateral resolution (x, y plane) until nanometric dimension. On the other hand, the sample height (z plane) can achieve sub angstrom resolution based on finely tuning interaction force between the tip and sample. Depending upon samples properties, scanning can be done continuously (contact mode) or intermittently (tapping mode) for hard or soft samples respectively (for more details see Shahin and Barrera, 2008).

AFM AND BIOLOGICAL MEMBRANES

AFM has long been used to visualize lipid bilayers with a height resolution near to 0.1 nm (Mou et al., 1995; Dufrene et al., 1997; Grandbois et al., 1998; Hollars and Dunn, 1998; Rinia et al., 1999;

McKiernan et al., 2000; Reviakine et al., 2000; Muresan and Lee, 2001) which has allowed dynamical detection of microdomains (rafts) in lipid bilayers and in native membranes *in vitro* (Dufrene et al., 1997; Giocondi et al., 2000, 2001, 2004; Yuan et al., 2002; Lawrence et al., 2003; Anderton et al., 2011). For example, using AFM (Lawrence et al., 2003) have studied in real time the effects of manipulating cholesterol levels in supported model membranes containing dioleoylphosphatidylcholine (DOPC) and sphingomyelin (SM). In absence of cholesterol, these membranes form small SM domains, which increase after cholesterol addition. An ordered and unique lipid raft domain is present at very high cholesterol concentrations, effect that is reversed once methyl-ß-cyclodextrin (MßCD, cholesterol chelator) is applied. In addition, time-lapse AFM has been used to visualize dynamical processes in living cells, like extension and retraction of lamellipodium in MCF-7 cells (Li et al., 2013). Recent AFM studies on native membranes have proved the presence of lipid rafts in erythrocytes with a size of 100–300 nm and irregular shape and height of 2–4 nm above membrane bilayer (Cai et al., 2012). Orsini et al. (2012) have shown detergent-resistant membranes (DRMs) in human breast cancer cells with sizes of 100–500 nm and heights 1–2 nm above the PM. Furthermore, they demonstrated the presence of flotillin-1, a specific raft marker. Altogether, these evidence highlight the usefulness of the AFM technique in the lipid rafts analysis.

MASS SPECTROMETRY OF MEMBRANE PROTEINS AND LIPID RAFTS

MS determines both abundance and precise mass of biomolecules based on their ionization and mass/charge relationship in the gas phase (Barrera and Robinson, 2011). MS has emerged as a powerful tool to quantitatively analyse complex phospholipids such as those contained in lipid rafts, including glycerophospholipids and sphingolipids, from crude extracts (Pulfer and Murphy, 2003; Han and Gross, 2005). Interestingly, apart from proteomics information, MS has identified intact membrane proteins (Barrera et al., 2013), and also the stoichiometry and nature of lipids bound to them (Barrera et al., 2008, 2009). Altogether these data have shown that MS can provide structural aspects all the way through proteomics and lipidomics to stoichiometries of intact complexes.

A decade ago, a proteomic study identified 238 PM proteins from *Arabidopsis thaliana* (Alexandersson et al., 2004). They found 114 integral/GPI and 124 peripheral proteins; however only 180 out of the total proteins detected were classified as having a known function.

Lipid rafts in plants were suggested by the presence of a Triton X-100 insoluble PM fraction or DRM in tobacco cells. This fraction exhibited a different protein composition to that of PM, including GPI-anchored proteins (Peskan et al., 2000). Other proteins associated to DRMs comprise receptor-like kinases (RLKs), G-proteins (Morel et al., 2004), redox system proteins (Lefebvre et al., 2007) and stress associated proteins (Cacas et al., 2012). A proteomic strategy was developed to characterize membrane proteins associated to sterol containing DRMs fractions in *A. thaliana* (Kierszniowska et al., 2009). They found a considerable number of GPI-anchored proteins and other proteins with unknown function. Remorin protein, a molecular marker for lipid rafts in plants, has also been localized in the PD in *Solanaceae* family (Raffaele et al., 2009) In agreement to this, Fernandez-Calvino et al. (2011), via proteomics of the PD in *A. thaliana*, reported a variety of GPI-anchored proteins and remorin. Altogether, these data suggest that lipid rafts may constitute the PD.

Apart from lipid rafts, other cell membrane domains are tetraspanin-enriched microdomains (TEMs) (Hemler, 2005). Tetraspanins are integral transmembrane proteins which contain four transmembrane domains and two extracellular loops. Tetraspanins associates with cholesterol through a palmitate (S-acylation of the protein), and with gangliosides (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Ono et al., 2001; Hemler, 2003, 2005). Most of their functions are involved in cell adhesion (to the extracellular matrix, other cells and pathogens), intercellular communication, membrane fusion and intracellular signaling. TEMs might enhance these processes by clustering functionally related molecules or by tightly packing a critical number of specific receptors at the PM (Yáñez-Mó et al., 2009). Lipid rafts and TEMs have similarities such as cholesterol enrichment (Le Naour et al., 2006) and localization in DRMs (Charrin et al., 2003). In contrast to lipid rafts, TEMs are mostly soluble in stronger non-ionic detergents, and resistant to cholesterol depletion (Claas et al., 2001), although partial disruption may be occasionally observed (Charrin et al., 2003). GPI-anchored proteins have not been detected in TEMs (Hemler, 2005). Based on tetraspanin identification and the absence of significant amounts of PM or endoplasmic reticulum markers in the PDs (Fernandez-Calvino et al., 2011), these nanopores could be constituted by highly specialized membrane microdomains that may contain TEMs. Indeed, it has been demonstrated the coalescence of lipid rafts and TEMs in human immunodeficiency virus type 1 (HIV-1) assembly sites on the PM by Förster resonance energy transfer (FRET) assay in living cells (Hogue et al., 2011). Furthermore, integrin-tetraspanin signaling complexes are partitioned into specific microdomains proximal to cholesterol-rich lipid rafts (Berditchevski, 2001). **Table 1** shows a summary of PD associated proteins with structural properties identified or hypothesized.

Mongrand et al. (2004) analyzed the lipidomics of DRMs isolated with Triton X-100 from tobacco PM. These microdomains mostly contained a sphyngolipid, named glucosylceramide (GluCer), and sterols such as stigmasterol, sitosterol, 24-methylcholesterol, and cholesterol. Using TLC and gas chromatography/mass spectrometry (GC/MS), two phosphoinositides PI(4)P y PI(4,5)P2 were quantified in DRMs of PM from tobacco and BY-2 cells (Furt et al., 2010). Both phosphoinositides represent less than 5% of total lipids in tobacco PM; however its relative amount is increased 11 times in membrane rafts. In addition, structural phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid were not abundant in DRMs compared to the PM (Mongrand et al., 2004). Although MS has advanced the knowledge of lipid composition in membrane rafts (Mongrand et al., 2010), its application on the study of PD is still absent.

Table 1 | Proteins localized in the PD.

Protein/Organism	Method	Mass (Da)/AGI code	X-ray structure homolog	Probable stoichiometry	Function	References
Myosin VIII-A *A.thaliana*	Immunolocalization	130007.5/At1g50360	–	Dimer	ATP binding, motor activity	Reichelt et al., 1999; Golomb et al., 2008
Calreticulin *Maize*	Immunolocalization	48527/At1g56340	3O0V, 3O0W, 3O0X Kozlov et al., 2010; 3POS, 3POW Chouquet et al., 2011	Monomer	Chaperonin promoting folding, oligomeric assembly and quality control in the ER; interaction with TMV MP	Baluska et al., 1999; Chen et al., 2005
Pectin methyl esterase or PME *N. tabacum*	Immunolocalization	64148.6/At1g53840	–	–	Catalyze esterification of pectins; specifically binds to the TMV MP	Dorokhov et al., 1999; Chen et al., 2000
Class III peroxidase *L. esculentum*	Transmission-electron microscopy	39559.0/At1g71695	1SCH (Schuller et al., 1996)	–	Production de hydroxyl radicals	Ehlers and Van Bel, 2010
Beta-1,3-Glucanase (AtBG_ppap) *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	45357.4/At5g42100	4GZI (Wojtkowiak et al., 2013)	Monomer	Degradation of callose; glycoside hydrolases; GPI-anchored PM protein	Bayer et al., 2006; Levy et al., 2007
Plasmodesmata Located Protein (PDLP) *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	32606.6/At5g43980	–	–	Membrane receptor type 1	Bayer et al., 2006; Thomas et al., 2008
Plasmodesmal Callose Binding (PDCB) *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	20364.4/At5g61130	–	–	GPI-anchored PM protein	Bayer et al., 2006; Simpson et al., 2009
LRR RLK *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	114874/At1g56145	3BEL (Xu et al., 2008)	Homodimer	Signaling	Walker, 1994; Fernandez-Calvino et al., 2011
Tetraspanin (TET3) *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	31887.9/At3g45600	1G8Q (Kitadokoro et al., 2001)	Homodimer- Heterodimer Boavida et al., 2013	Formation of membrane microdomains	Silvie et al., 2006; Espenel et al., 2008; Fernandez-Calvino et al., 2011
crRLK1L *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	91822.4/At5g24010	3BEL (Xu et al., 2008)	Homodimer	Signaling	Walker, 1994; Fernandez-Calvino et al., 2011
S-domain RLK *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	96464.9/At4g21380	3BEL (Xu et al., 2008)	Homodimer	Signaling	Walker, 1994; Fernandez-Calvino et al., 2011

(Continued)

Table 1 | Continued

Protein/Organism	Method	Mass (Da)/AGI code	X-ray structure homolog	Probable stoichiometry	Function	References
Beta-1,6-N-acetylglucosaminyl transferase-like enzyme (AtGnTL) *A. thaliana*	Fluorescent fusion protein via confocal microscopy	39516.5/At3g52060	2GAM (Pak et al., 2006)	–	Glycosyltransferase	Zalepa-King and Citovsky, 2013
Remorin *S. lycopersicum*	Immunolocalization	20968/At2g45820	–	Homotrimer	–	Raffaele et al., 2009; Perraki et al., 2012
Actin *C. corallina*	Immunolocalization	41735.4/At5g09810	–	–	Cytoskeleton protein	Blackman and Overall, 1998
LYM2 *A. thaliana*	Proteomics/fluorescent protein fusion/confocal microscopy	37721,6/At2g17120	–	–	GPI-anchored PM protein; pattern-recognition receptor of pathogen	Fernandez-Calvino et al., 2011; Faulkner et al., 2013

This table is not an exhaustive list of all proteins that have been associated with PD.

RLK, ReceptorLike Kinase; LRR RLK, Leucine Rich Repeat Receptor –Like Kinases; crRLK1L, Catharanthus roseus Receptor-Like kinase1-like; DUF, Domain of Unknown Function; TMV MP, movement protein encoded by tobacco mosaic virus; LYM2, Lysin motif domain-containing glycosylphosphatidylinositol-anchored protein 2.

CHARACTERIZATION OF THE PD MEMBRANE VIA AFM AND MS

It has been widely accepted that complementary structural techniques including AFM and MS are needed to understand and predict the behavior of intact membrane proteins (Barrera and Edwardson, 2008; Barrera and Robinson, 2011). Additionally, AFM has proved to represent an excellent and unique choice to visualize the dynamics of lipid rafts (Henderson et al., 2004). In this perspective article we propose a combination of these methods to get novel insights into the protein and lipid composition of the PD, in particular to determine the presence of lipid rafts, membrane protein stoichiometry and lipidomics. Considering that PD is a dynamic structure that responds to environmental stimuli, probably by changing its protein composition (Maule, 2008), we propose to evaluate *in vitro* purified PD vesicles under different physiological conditions. As stated in **Figure 1A**, Fernandez-Calvino et al., 2011 reported a methodology to purify PD vesicles, which are derived from membrane fractions without significant cell wall according to electron microscopy imaging. Further analysis by immunoblot of the samples confirmed the presence of PD proteins like PDLP1, and the absence of proteins associated to ER (BiP), Golgi (Membrine 11) and chloroplast (thylakoid P16).

Working with purified PD vesicles under physiological conditions (**Figure 1A**), AFM imaging could be applied to identify lipid rafts and TEMs based on topological parameters. Expected heights above plasma membrane for lipid rafts and TEMs are 1–4 nm (Cai et al., 2012; Orsini et al., 2012) and 5–6 nm (Brisson et al., 1983; Taylor and Robertson, 1984; Walz et al., 1995; Min et al., 2002, 2003), respectively. A putative AFM imaging of the PD vesicles is shown in **Figure 1B**. PM areas correspond to the minimal height of the vesicles (green color) which should be composed mostly by phospholipids. Lipid rafts correspond to flat domains of 1.5–2 nm above plasma membrane (yellow color). TEMs correspond to flat domains of 5–6 nm above plasma membrane (red color). Using MßCD on PD vesicles we could trigger a reorganization of the lipid rafts but not affecting TEMs (Claas et al., 2001; Giocondi et al., 2004) which would allow us to differentiate both membrane domains. Membrane proteins should be also observed in some areas of the lipid rafts and a variety of hypotheses could be tested. For example, remorin has been proposed as lipid raft molecular marker and has been found in the PD (**Table 1**). AFM imaging of this protein, based on its molecular weight, would induce a particle height less than 1 nm above the lipid raft (**Figure 1B**, upper panel). Remorin can also form homotrimers *in vitro* where each subunit is anchored to the membrane via C-terminal tails (Perraki et al., 2012). Therefore, assuming remorin (arrows, **Figure 1B**) can present different stoichiometries in the lipid rafts, AFM imaging could detect monomers (**Figure 1B**, ii) as well as trimers (**Figure 1B**, i) over the lipid raft flat surface. This is based only on approximate changes on molecular area or volume between both structures. As other proteins can be present in this membrane domain, another strategy is needed to corroborate the presence of this protein, which is force spectroscopy, using AFM tip functionalized (Dufrêne et al., 2013) with a specific anti-remorin antibody (Lefebvre et al., 2010). This technique consists of chemical modifications of the

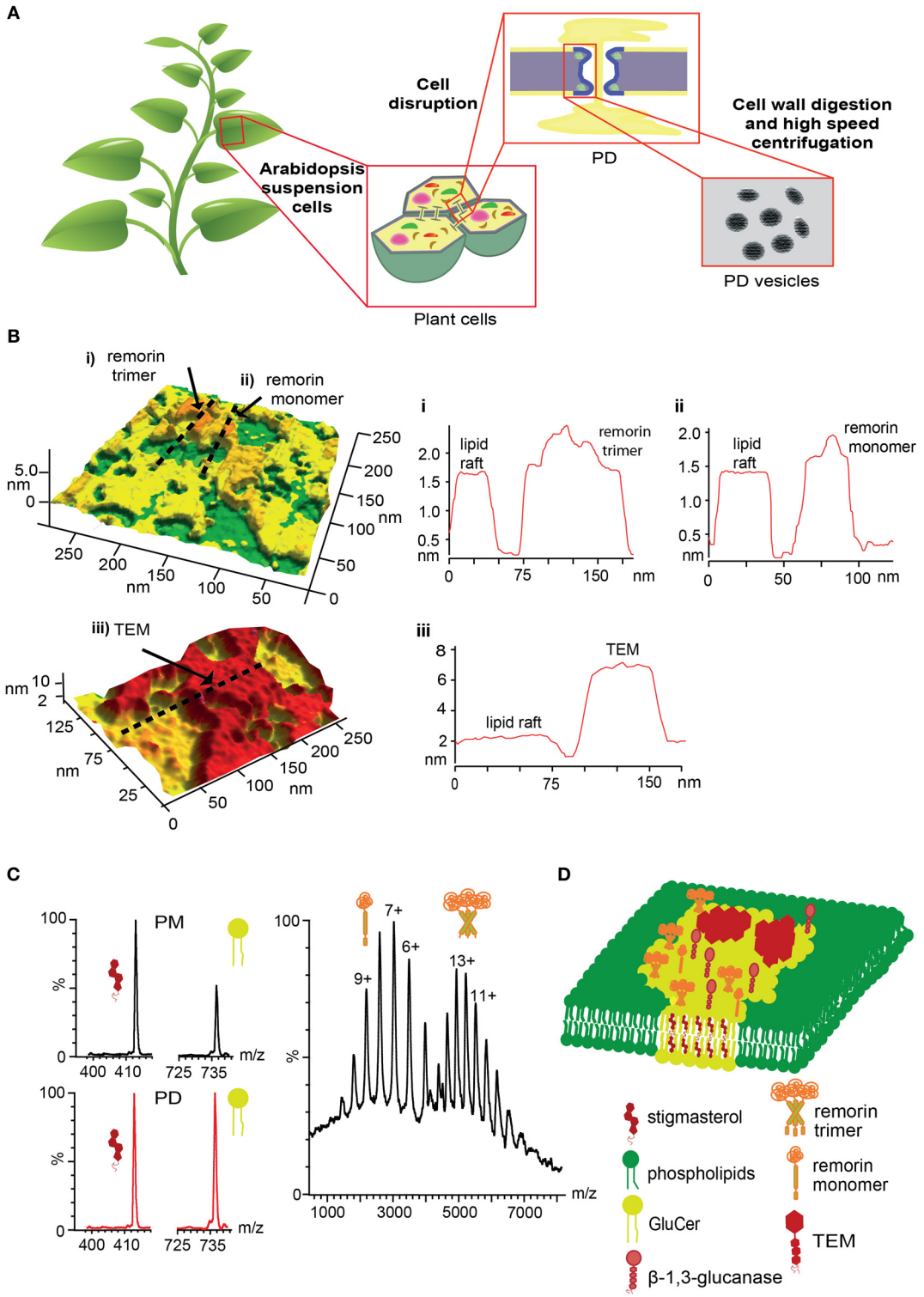

FIGURE 1 | Combined AFM and MS methodology to characterize the PD protein and lipid structure. (A) Scheme of the purification of PD vesicles from *A. thaliana* suspension cells. **(B)** AFM imaging simulations of lipid rafts, TEMs and remorin in PD membrane. Upper panel, AFM imaging of PM (green) and lipid rafts (yellow) where remorin (arrows) monomer and trimers can be localized above lipid raft domains. Lower panel, AFM imaging of lipid rafts and TEMs (red) is graphed. Right panels show a selection of cross section analyses **(i–iii)** for lipid and protein areas indicated as dashed lines in left panels. **(C)** Mass spectra simulations of stigmasterol and GluCer lipids from PM and PD are shown in black and red lines respectively (left panel). Mass spectra simulations of intact remorin showing monomeric and trimeric stoichiometries (right panel). **(D)** PD membrane model based on AFM and MS results.

AFM tip to make a covalent bond between the tip surface and a protein/biomolecule (probe). In consequence this probe can interact specifically with the sample. In this case, at the single molecule event, a significant binding affinity on the remorin/anti-remorin antibody complex would trigger an increase of the interaction force that subsequently can be transformed in dissociation constant (Le et al., 2011) for the remorin-antibody binding reaction. This figure can then be compared with traditional experiments to analyse binding such as isothermal calorimetry or surface plasmon resonance, usually used for much larger amount of sample. As stated previously, GPI-anchored proteins have been also proposed as lipid raft molecular markers in mammalian and plant cells (Sangiorgio et al., 2004; Mongrand et al., 2010) and therefore ß- 1,3-glucanase, localized in the PD (**Table 1**), is another alternative to study protein localization in the lipid rafts via AFM. Despite technical advances, visualization of PDs in living cells by AFM is currently precluded by the PD location, in the cell-to-cell physical communication that is not accessible to the AFM tip.

To characterize the lipidomics of PD vesicles we could use Liquid Chromatography-MS/MS. Based on Mongrand et al. (2004), it is expected that GluCer would be distributed in DRMs rather than in PM with a peak at 736.6 m/z. On the other hand, stigmasterol (peak at 412.7 m/z) would be in similar proportions in PM and lipid rafts. **Figure 1C** shows mass spectra of both lipid species. Hence MS of PM lipids would show a larger stigmasterol/GluCer intensity ratio (black spectra in **Figure 1C**) compared to a MS of PD lipids (red spectra in **Figure 1C**) from plant cell samples. As recently demonstrated, MS has been used to determine the stoichiometry of intact membrane protein complexes as well as to identify post-translational modifications and small molecules bound to membrane proteins. This can be done by removing the protective micelle environment via collision-induced dissociation with neutral gas molecules inside mass spectrometer that results in the releasing of intact membrane proteins (Barrera and Robinson, 2011; Barrera et al., 2013). Using MS on solubilized PD vesicles, it would be possible to determine the stoichiometry of remorin and test whether or not form monomers and/or trimers (**Figure 1C**), and therefore corroborate the data obtained by AFM imaging (see above). The measurement can be very accurate within Da resolution. In addition, the same sample could be digested by trypsin in proteomic experiments to verify possible protein modifications.

In our experimental design we have proposed to work under different physiological conditions to analyse variations on PD constitution and conformation in response to environmental stimuli. It is reported that changes on PD permeability by pathogen infection have been observed after increasing callose deposition on the cell wall near to the PD neck, which reduces channel diameter (Zavaliev et al., 2011). In addition, the cellular redox state regulates PD permeability. Mutations in mitochondrial RNA helicase, *ise1*, (Stonebloom et al., 2009), and thioredoxin type m3, *gat1*, (Benitez-Alfonso and Jackson, 2009), induce an increase of reactive oxygen species (ROS) in plant cells. However, both mutants have opposite effects on the PD permeability, while *ise1* increases permeability, *gat1* decreases it. More recently, H_2O_2 treatments display a byphasic effect on PD permeability, where 0.6 mM and 6 mM H_2O_2 produce a two-fold increment and total abolition, respectively (Rutschow et al., 2011). These results confirm that redox state controls the PD permeability and makes it an important candidate to modulate the lipid and protein abundance in the PD. Therefore, we propose to incubate cultured plant cells with low (0.6 mM) and high (6 mM) H_2O_2 concentrations prior to PD vesicles purification. There is evidence showing that the plasmodesmal aperture is regulated by callose deposition at the neck region (Simpson et al., 2009; Zavaliev et al., 2011). Therefore, an increase in permeability induced by treatment with low concentrations of H_2O_2 could be mediated in part by the increase in the abundance of β-1, 3 -glucanase (protein that degrades callose) and/or a decrease in the abundance of plasmodesmal callose binding protein (PDCB1). It has been shown that PDCB1 overexpression augmented callose accumulation resulting in a reduction of green fluorescent protein (GFP) diffusion. Therefore, there is an association between PDCB-mediated callose deposition and plant cell-to-cell communication (Simpson et al., 2009). An opposite effect would be observed after high H_2O_2 concentrations. Interestingly, protein clustering in lipid rafts depends on cholesterol presence (Simons and Toomre, 2000). Moreover oxygenated derivatives of cholesterol (oxysterols) can be generated by ROS (Terao, 2014) and trigger a dynamic redistribution of lipids from lipid rafts (Bacia et al., 2005). For example, if stigmasterol is similarly modified, an oxysterol signal would appear in the MS lipidomics analysis and probably would affect the size and dynamics of the lipid raft imaged by AFM. These evidence suggest that cellular redox states may change PD permeability thorough lipid and protein modifications, which can be studied by traditional lipidomics and proteomics in MS experiments.

These proposed experiments would allow us to postulate a structural PD membrane model (**Figure 1D**) where specific lipid and protein components are responsible for the mechanisms underlying biomolecule transport.

In this perspective article, we have discussed the potential use of complementary state-of-the-art AFM and MS to characterize the PD lipid and protein structure from native conditions. We envisage that novel studies in the near future combining this with plant genomics could lead to an integrative view on the PD role for cell-to-cell communication throughout plant development.

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Exploring the role of lipids in intercellular conduits: breakthroughs in the pipeline

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It has been known for more than a century that most of the plant cells are connected to their neighbors through membranous pores perforating the cell wall, namely plasmodesmata (PDs). The recent discovery of tunneling nanotubes (TNTs), thin membrane bridges established between distant mammalian cells, suggests that intercellular communication mediated through cytoplasmic continuity could be a conserved feature of eukaryotic organisms. Although TNTs differ from PDs in their formation and architecture, both are characterized by a continuity of the plasma membrane between two cells, delimiting a nanotubular channel supported by actin-based cytoskeleton. Due to this unusual membrane organization, lipids are likely to play critical roles in the formation and stability of intercellular conduits like TNTs and PDs, but also in regulating the transfer through these structures. While it is crucial for a better understanding of those fascinating communication highways, the study of TNT lipid composition and dynamics turned out to be extremely challenging. The present review aims to give an overview of the recent findings in this context. We will also discuss some of the promising imaging approaches, which might be the key for future breakthroughs in the field and could also benefit the research on PDs.

Keywords: intercellular communication, plasmodesmata, tunneling nanotubes, membrane lipids, phosphoinositides, imaging techniques

INTRODUCTION

The existence of "cytoplasmic bridges" between plant cells was first reported by Tangl (1880). These structures, later named "plasmodesmata" (PDs), are thin plasma-membrane lined pores embedded in the cell wall and allowing direct cell-to-cell transmission of materials and signals (Kragler, 2013). More recently, the discovery that many different mammalian cell types can also be connected by cytoplasmic bridges, namely tunneling nanotubes (TNTs; **Figure 1A**), suggests that this type of communication is not a hallmark of plant cells (Rustom et al., 2004; Rustom, 2009). Like PDs, TNTs are thin membranous channels supported by the actin cytoskeleton mediating intercellular communication through cytoplasmic continuity (**Figures 1B,C**). These structures are dynamic and heterogenous and contrary to other types of membrane protrusions, such as filopodia, do not touch the substrate in cell culture (Rustom et al., 2004; Abounit and Zurzolo, 2012). Although the lack of known molecular markers hampers the identification of TNTs within tissues, several recent studies described the presence of TNT-like structures *in vivo* (Chinnery et al., 2008; Pyrgaki et al., 2010; Lou et al., 2012; Seyed-Razavi et al., 2013). If TNT diameter (20–500 nm) is comparable to PD diameter (∼50 nm), TNT length is highly variable and can extend up to several cell diameters (∼100 μm), whereas the length of PD is determined by the cell wall thickness (Gerdes et al., 2007). Another difference between the two structures is that TNTs lack the central desmotubule (membranous rod of appressed endoplasmic reticulum), which is typical of most PDs (**Figures 1B,C**).

In addition, while primary PDs result from incomplete cell plate formation during cytokinesis, TNTs, like secondary PDs, are formed *de novo* and can be observed between heterotypic cells (Gerdes et al., 2007). Therefore TNTs are very dynamic structures which can be formed after cells previously in contact detach from one another, or can arise from the extension of filopodia-like protrusions toward neighboring cells (Abounit and Zurzolo, 2012; Kimura et al., 2012). Although some early steps in TNT genesis have been highlighted, the molecular pathways involved in their formation are still unclear (Marzo et al., 2012; Gousset et al., 2013). In addition, the structural (e.g., length/diameter, presence of microtubules, open-endedness) and functional (e.g., type of transferred cargoes/signals) diversity observed among TNT-like structures in various cell-types suggests that they may also differ in their formation mechanisms (Abounit and Zurzolo, 2012). A wide variety of cellular materials, such as cytoplasmic molecules, plasma membrane (PM) components, vesicles derived from various organelles, and even whole organelles (e.g., mitochondria) have been shown to transfer through TNTs (Marzo et al., 2012; Gerdes et al., 2013). Furthermore, TNTs can be "hijacked" by different pathogens, such as bacteria, viruses, or prions, and might represent a general way for pathogen spreading (Hurtig et al., 2010; Marzo et al., 2012). Therefore these structures attracted much attention in cell biology over the last decade. While some TNT constituents, such as actin and myosin which are also found in PDs, have been identified (Abounit and Zurzolo, 2012), the lipid composition of their membrane remains largely unknown. Nevertheless, this question is of major interest because the peculiar conformation of intercellular conduits like TNTs and PDs suggests that lipids play crucial roles in their establishment and function. Indeed, although lipids have for a long time been considered as

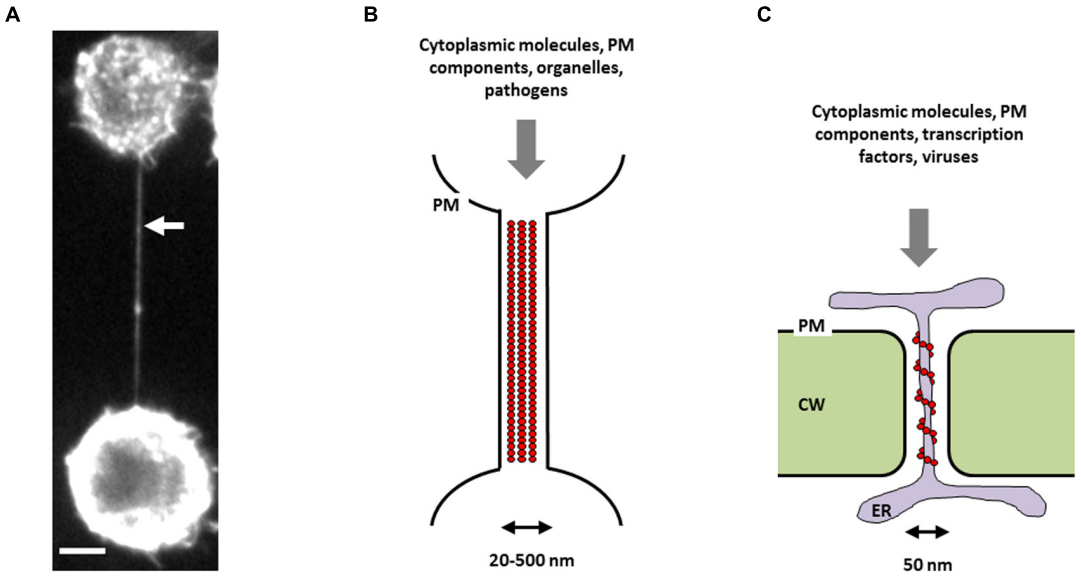

FIGURE 1 | Intercellular conduits in mammalian and plant cells. **(A)** Picture of a TNT connecting two neuronal CAD cells in culture. Cells were stained with wheat germ agglutinin in order to visualize TNT membrane, then fixed, and imaged by spinning-disk fluorescence microscopy. White arrow indicates a TNT connecting two remote cells. Scale bar = 5 μM. Schematic representations of a TNT **(B)** and a PD **(C)**. PM = plasma membrane, CW = cell wall, ER = endoplasmic reticulum, red circles = actin-based cytoskeleton. Note the absence of a midbody, which excludes the possibility that this structure could be an intercellular bridge.

passive building blocks of cellular membranes, their active role in many cellular processes such as membrane trafficking, cytoskeleton remodeling and signaling, is now widely recognized (Takenawa and Itoh, 2001; Wenk, 2005). Specifically, some membrane lipids, such as phosphoinositides or sphingolipids, can be precursors of signaling molecules and can also directly interact with proteins, thus regulating their activity or subcellular location (Wenk, 2005; Delage et al., 2013). In addition, lipids can segregate in membrane nano and microdomains such as rafts, "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" (Pike, 2006; Simons and Sampaio, 2011) involved in many biological events (Sonnino and Prinetti, 2013).

The present review aims to emphasize the multiple different functions that lipids might exert in TNTs and to summarize the current knowledge on this topic. In addition, we will discuss some of the promising imaging techniques that might be crucial to decipher lipid organization within nanotubular intercellular conduits such as TNTs or PDs.

MEMBRANE LIPIDS AS POSSIBLE KEYSTONES OF TNT STRUCTURE AND FUNCTION: THE PREMISES

It is noteworthy that, similar to PD membrane, the membrane delimiting TNTs is characterized by a strong curvature, which suggests that its lipid composition differs from the surrounding PM (**Figure 2A**). Indeed, different studies using artificial membrane nanotubes and theoretical predictions highlighted the reciprocal influence of membrane curvature and lipid segregation (Callan-Jones et al., 2011; Kabaso et al., 2012). Lipid sorting can reduce the energy cost of membrane bending, which depends on the deformability of the bilayer and on the molecular shape of its lipid components (Callan-Jones et al., 2011; Lokar et al., 2012). Interestingly, several recent papers indicated that the clustering of lipid and protein nanodomains with an affinity for highly curved membrane regions may induce a tubular budding of the membrane, even in absence of a pushing or pulling force from the cytoskeleton (**Figure 2B**; Farsad and Camilli, 2003; Gimsa et al., 2007; Iglic et al., 2007; Römer et al., 2007). The accumulation of specific membrane domains (or rafts) enriched in proteins that preferentially localize to cylindrical membrane protrusions and generate anisotropy, like the membrane protein prominin, could also be crucial for the stability of those structures (**Figure 2A**; Iglič, 2006; Veranič et al., 2008; Hurtig et al., 2010; Kabaso et al., 2012). In addition, an enrichment of ordered lipid domains could influence TNT transfer function by restricting the lateral diffusion of membrane components or by targeting membrane proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins, to TNTs for intercellular transfer (**Figure 2C**; Veranič et al., 2008; Tilsner et al., 2010). Interestingly, the presence of rafts in filopodia and other membrane protrusions has been highlighted (Corbeil et al., 2001; Huttner and Zimmerberg, 2001; Gupta and DeFranco, 2003). Furthermore several evidences pointing towards a possible enrichment of membrane rafts in PDs have been reported (Tilsner et al., 2010; Cacas et al., 2012) suggesting that this could be a common feature of thin tubular membrane structures.

Lipid constituents of the PM can also influence its curvature via their tight interplay with membrane-bending proteins,

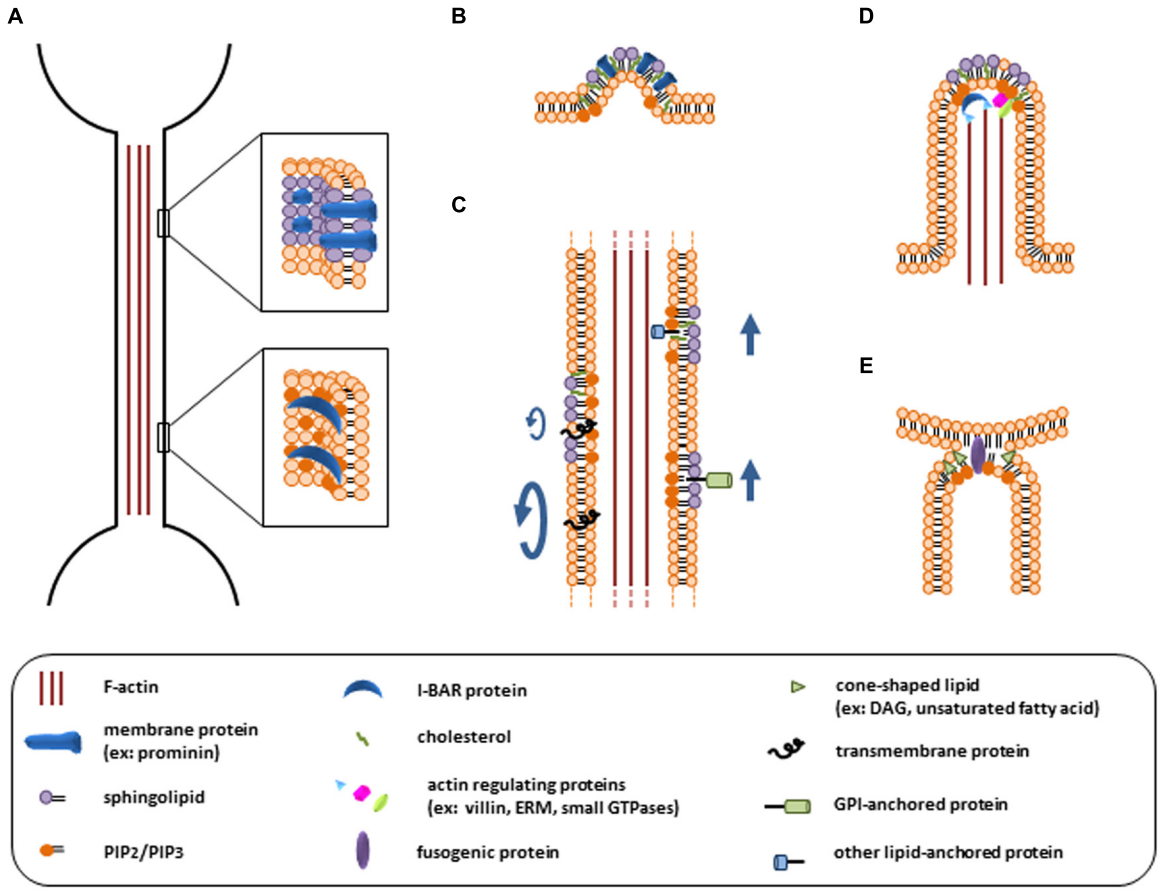

FIGURE 2 | Potential key roles of membrane lipids in TNT formation and function. (A) Stabilization of the highly curved TNT membrane by lipid and protein nanodomains such as rafts, or by I-BAR proteins recruited via phosphoinositide binding. **(B)** Induction of membrane tubular budding by lipid and protein clustering. **(C)** Influence of membrane organization on TNT transfer function. Ordered lipid domains can restrict the lateral diffusion of membrane proteins (*left*) or promote their targeting and subsequent transfer through TNTs (*right*). **(D)** Regulation of actin cytoskeleton by phosphoinositides during TNT formation via filopodia-like protrusions. **(E)** Facilitation of membrane fusion by lipid physical properties.

such as Bin/amphiphysin/Rvs (BAR) domain-containing proteins (**Figure 2A**; Rao and Haucke, 2011). In contrast to other members of the BAR domain family which generate membrane invaginations, inverse BAR (I-BAR) domains recognize negative curvature and induce membrane protrusions (Saarikangas et al., 2009). I-BAR proteins have been implicated in filopodia generation in different cell types (Millard et al., 2005; Mattila et al., 2007; Saarikangas et al., 2009) and might play a similar role in TNT formation. The I-BAR domain electrostatically interacts with negatively charged phospholipids, with a stronger affinity for phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] (**Figure 2A**; Mattila et al., 2007; Saarikangas et al., 2009). Interestingly, I-BAR proteins can also bind many different regulators of the actin cytoskeleton and their main role in filopodia formation may be achieved through the coupling of membrane protrusion and actin filament formation (**Figure 2D**; Ahmed et al., 2010).

The structural and functional relation between the actin cytoskeleton and lipid constituents of the PM is far from being limited to I-BAR proteins. It has been suggested that components of the actin cytoskeleton exert an ordering effect on the lipid bilayer and could contribute to assemble membrane rafts (Chichili and Rodgers, 2009; Gowrishankar et al., 2012). Various raft structural and functional features require an intact actin cytoskeleton. In turn, proteins and lipids involved in cytoskeleton regulation or anchorage to the PM are found associated with rafts (**Figure 2D**). Notably, the inner leaflet of membrane rafts is enriched with phosphoinositides, such as $PI(4,5)P_2$ and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which emerged as major regulators of cytoskeleton structure and dynamics (Saarikangas et al., 2010). In a nutshell, phosphoinositides positively regulate proteins that promote actin polymerization and inhibit proteins that induce filament disassembly (**Figure 2D**). They also contribute to anchor actin filament to the PM through protein linkers, such as ezrin-radixin-moesin (ERM) proteins, key proteins in the formation of PM protrusions (Chichili and Rodgers, 2009; Saarikangas et al., 2010). Specifically, the role of $PI(4,5)P_2$ and PIP_3 in filopodia formation, in relation with the actin cytoskeleton, has been widely documented (Arjonen et al., 2011; Khurana and George, 2011). Interestingly, sphingosine 1-phosphate can

also induce filopodia formation through the activation of ERM proteins (Gandy et al., 2013). Because actin plays a major role in TNT generation via filopodia-like protrusions (Abounit and Zurzolo, 2012; Gousset et al., 2013), we speculate that phosphoinositides and sphingolipids are likely to be involved in this process.

Another crucial step of TNT formation in which lipids may play an important role is the fusion of the two plasmalemmas, leading to cytoplasmic continuity of the connected cells. Indeed, although fusion probably requires fusogenic proteins it is sensitive to membrane lipid composition. Depending on their intrinsic shape and their position in the inner or outer leaflet of the bilayer, lipids may differently affect the propensity of the membranes to merge. For example, lipids with an inverted cone shape, such as phosphoinositides or lysophospholipids, can promote the fusion of membranes when located in the distal leaflet, whereas cone-shaped lipids, like cis-unsaturated fatty acids or diacylglycerol, promote it when located in the proximal leaflet (**Figure 2E**; Chernomordik and Kozlov, 2003; Larijani and Poccia, 2012).

MEMBRANE LIPIDS AS POSSIBLE KEYSTONES OF TNT STRUCTURE AND FUNCTION: THE CURRENT KNOWLEDGE

In accordance with what has been hypothesized from theoretical studies, experimental data suggested the presence of specific lipid domains in the membrane lining TNTs. Indeed, by using the raft marker ostreolysin (Oly) Iglič and coworkers recently highlighted the presence of cholesterol-sphingomyelin nanodomains within TNTs in a T24 urothelial cancer cell line (Lokar et al., 2012). In contrast to Oly, very little binding of cholera toxin B, which binds to the raft-specific ganglioside GM1, was observed along TNTs, and immunofluorescence studies do not reveal the presence of caveolin-1 and flotillin-1 raft markers (Bickel, 2002). On the other hand, addition of the cholesterol depletion agent methyl-β-cyclodextrin and the growth in cholesterol-free medium were shown to reduce the number of TNTs, suggesting a role for cholesterol in the stability of these structures (Lokar et al., 2012). Cholesterol is expected to be more present in the external leaflet of TNT to minimize the bending energy cost. According to their experimental results and computational model, the authors proposed that cholesterol depletion from the outer leaflet may reduce the area difference between the two leaflets, thus favoring more planar conformation and leading to the detachment of TNT from the parent PM (Lokar et al., 2012).

To our knowledge, no other data regarding the lipid composition of TNTs has been reported in the literature thus far. However, indirect evidences support the role of PIP_3 in TNT formation. Wang and collaborators (Wang et al., 2011) recently highlighted the involvement of the Akt/phosphatidylinositol 3-kinase (PI3K)/mTor pathway in TNT generation in astrocytes under H_2O_2 treatment. Interestingly, they observed a drastic reduction of stress-induced TNT formation in astrocytes treated with PI3K and mTor inhibitors or expressing the Akt dominant negative mutant, whereas expression of the constitutive form of Akt increased the number of connections. They also reported an increase of Akt and PI3K phosphorylated forms upon H_2O_2 treatment. In this context, PIP_3 formation thus appears as an important step for TNT genesis.

In addition, recent data obtained in our laboratory using CAD cells, a mouse neuronal cell line of catecholaminergic origin, also suggested the importance of PIP_3 for TNT generation (Gousset et al., 2013). It was shown that expression of the unconventional molecular motor myosin 10 (Myo10) increases the number of functional TNTs and the vesicle transfer between connected cells. A point mutation in the second pleckstrin homology of Myo10, which impairs its binding to PIP_3, hindered the ability of Myo10 to induce TNT formation. Thus, in accordance with what has been reported for filopodia (Plantard et al., 2010; Lu et al., 2011), Myo10 recruitment to the PM through PIP_3 binding seems to be necessary for Myo10 role in TNT induction. However, contrary to what has been shown for astrocytes, no correlation between Akt activation and TNT formation has been observed in CAD cells, suggesting a PI3K dependent but Akt independent pathway (Gousset et al., 2013).

PROMISING IMAGING TECHNIQUES FOR FURTHER BREAKTHROUGHS

Although the cues for a critical role of lipids in TNT are considerable, research in the field has been hampered by technical limitations. While PD-enriched fractions can be obtained from plant cell walls, thus allowing biochemical approaches (Maule et al., 2011), isolation of "bona-fide" TNT from the cell bodies is quite challenging. Indeed, although the isolation and proteomic study of bridging conduits connecting macrophages has been recently reported (Kadiu and Gendelman, 2011), the technique used to harvest those thicker and chemotaxis-driven protrusions does not appear to be applicable to the isolation of canonical TNTs. On the other hand, imaging techniques are to date the most suitable approaches to study TNT components. As a sensitive and versatile technique, and because it allows to image live cells, fluorescence microscopy is particularly well-suited to investigate lipid organization in the membrane of intercellular conduits.

While classical studies consist of imaging the distribution of the fluorescence intensity from a given fluorophore (lipid-binding probes, lipid analogs, or fluorescently tagged proteins used as raft markers; Hullin-Matsuda et al., 2009), several recent techniques made it possible to monitor additional fluorescence parameters such as spectral shifts and anisotropy (Braeckmans et al., 2011; Bastos et al., 2012). Combined with the utilization of probes whose physical properties are influenced by membrane packing order, such as 2-dimethylamino-6-lauroylnaphthalene (Laurdan), these techniques could be valuable to investigate the presence of lipid rafts in intercellular conduits (Dodes Traian et al., 2011; Owen et al., 2011). Because membrane organization can influence probe fluorescence lifetimes, this question could also be addressed by fluorescence lifetime imaging microscopy (FLIM; Bastos et al., 2012). Furthermore FLIM coupled with Förster resonance energy transfer (FRET), FLIM-FRET, can be applied to cellular membranes to address major questions, like the formation of lipid domain clusters, probe partition between specific membrane domains, or interaction of membrane components within a given domain (de Almeida et al., 2009; Stöckl and Herrmann, 2010). This technique has been successfully used by Konig et al. (2008) to demonstrate the association of the cortical actin meshwork with PIP_3-enriched compartments of the PM. Therefore a similar approach could

yield important information on lipid/actin interactions within intercellular conduits.

Because lateral organization of the membrane affects the mobility of its constituents, sub-resolution membrane domains can also be analyzed thanks to fluorescence microscopy techniques based on molecular dynamics assessment (Owen et al., 2009). This include highly sensitive single molecule techniques like fluorescence correlation spectroscopy (FCS) and single particle tracking (SPT), which could allow to address crucial questions regarding membrane domains, such as lipid–lipid and lipid–protein interactions within the tubular membranes, which cannot be unraveled with conventional optical methods (Chiantia et al., 2009; Owen et al., 2009).

A major issue when studying lipid organization in cellular membranes is that the predicted size of lipid nanodomains is below the resolution limit of classical optical microscopes (Pike, 2006; Owen et al., 2012). The development of super-resolution techniques that allow to image structures beyond the diffraction limit, such as photo-activated localization microscopy (PALM), stimulated emission depletion (STED) microscopy, and structured illumination microscopy (SIM), greatly improved the possibilities in the field. Combinations of far-field super-resolution techniques with approaches such as FLIM, FCS, and SPT have substantially increased our perception of lipid organization in biological membranes during the past few years (Owen et al., 2009) and may represent the most promising way to decipher lipid organization and dynamics within TNTs or PDs.

An alternative to fluorescence microscopy for high-resolution study of membrane organization within intercellular conduits might be the utilization of scanning or transmission electron microscopy (EM; Rustom et al., 2004; Lokar et al., 2010). However, two major hurdles in this context are the impossibility to observe live cells and the difficulties to preserve both the fragile nanotubular structure and lipid distribution in the membrane (Rustom et al., 2004; Bell and Oparka, 2011; Sonnino and Prinetti, 2013). Indeed, EM requires multiple preparation steps susceptible to alter cellular structures and to generate artifacts. Development of fixation procedures allowing a better preservation of the structures, such as high pressure freezing, may overcome some of these issues (Vanhecke et al., 2008). EM can be coupled with fluorescence imaging studies, thus enabling to combine contextual information obtained by fluorescence microscopy in live cells with the resolution of EM but also to study dynamic processes or rare events and/or structures (McDonald, 2009). Such correlative approaches offer very interesting perspectives in the study of TNT and PD formation and transfer function and are the most promising to answer still unresolved questions on the structural/functional diversity of the various TNT-like structures described to date (cf. Introduction, Abounit and Zurzolo, 2012).

Finally, because separating TNTs from the cell constitutes a technical challenge that may be difficult to overcome, an alternative non-targeted approach to resolve lipid distribution in intercellular conduits may come from the developments of imaging mass spectrometry techniques (IMS; Ellis et al., 2013). Although the current technical limitations of IMS are critical for the study of very thin and fragile structures like TNTs, research towards improvement in achievable resolution/sensitivity and in sample preparation procedures is very active, quickly expanding the possibilities of these techniques (Passarelli and Winograd, 2011; Passarelli and Ewing, 2013).

PERSPECTIVES

Despite differences in their formation and architecture, TNTs and PDs present striking functional and structural similarities, which need to be thoroughly explored. The questions raised by their unusual nanotubular membrane conformation are largely overlapping; therefore data obtained on TNT can be informative on PD membrane and vice versa. In these review we have underlined the importance of studying the lipid composition and dynamics in these structures, as they are likely to be key elements regulating their structure and function. In addition to the imaging techniques described above, biophysical approaches and computational models may also greatly contribute to extend our knowledge on this important subject. As it is often the case for emerging fields, we believe that the key for a better understanding of those fascinating intercellular communication highways lies in pushing the current technology to new applications and in the development of transkingdom and interdisciplinary studies.

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Arabidopsis mutants in sphingolipid synthesis as tools to understand the structure and function of membrane microdomains in plasmodesmata

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Plasmodesmata—intercellular channels that communicate adjacent cells—possess complex membranous structures. Recent evidences indicate that plasmodesmata contain membrane microdomains. In order to understand how these submembrane regions collaborate to plasmodesmata function, it is necessary to characterize their size, composition and dynamics. An approach that can shed light on these microdomain features is based on the use of *Arabidopsis* mutants in sphingolipid synthesis. Sphingolipids are canonical components of microdomains together with sterols and some glycerolipids. Moreover, sphingolipids are transducers in pathways that display programmed cell death as a defense mechanism against pathogens. The study of *Arabidopsis* mutants would allow determining which structural features of the sphingolipids are important for the formation and stability of microdomains, and if defense signaling networks using sphingoid bases as second messengers are associated to plasmodesmata operation. Such studies need to be complemented by analysis of the ultrastructure and the use of protein probes for plasmodesmata microdomains and may constitute a very valuable source of information to analyze these membrane structures.

Keywords: sphingolipid *Arabidopsis* mutants, sphingolipids and microdomains, long chain bases, sphingoid bases, microdomains and plasmodesmata

INTRODUCTION

Plasmodesmata (PD) are specialized membranous structures that allow the communication among contiguous plant cells, originating interconnected symplastic domains. Communication arise through these intercellular pores that allow the exchange of small molecules, such as ions, sugars, phytohormones and macromolecules -RNA, transcription factors, even virus (Kim and Zambrisky, 2005) and effectors derived from pathogens (Lewis et al., 2009). This selective intercellular flow of molecules follows a defined direction and occurs at precise developmental stages or during stress responses (Kragler, 2013).

Imaging techniques that allow preservation of PD structure revealed a very complex and refined organization, but its molecular composition is difficult to dissect by biochemical approaches (Brunkard et al., 2013; Salmon and Bayer, 2013). However, PD are stable assemblies that can be found in cell wall preparations (Brecknock et al., 2011; Salmon and Bayer, 2013) and can even survive treatments involving cell autophagy (**Figure 1**). PD are formed by the extension of the PM of two adjacent cells, containing a central cylinder constituted by the prolongation of the endoplasmic reticulum (ER) of the joint cells. This ER is embedded in a cytoplasmic milieu common to the interconnected cells. Insoluble glycans as callose are deposited in the neck of the structure (Maule et al., 2011).

While many of the proteins present in the PD are known (Fernandez-Calvino et al., 2011; Raffaele et al., 2009), few studies have dealt with the lipid phase from PD (Cacas et al., 2012). Recent evidences suggest the presence of membrane microdomains in the PM of the PD (Tilsner et al., 2013). Remorin, a key protein identified and considered a marker of plant PM microdomains is present in PD (Raffaele et al., 2009; Mongrand et al., 2010). Moreover, glycosylphosphatidylinositol (GPI) anchored proteins frequently found in PM microdomains (Brown and Rose, 1992; Schroeder et al., 1994) have been localized in the PD through subcellular fractionation and proteomic analysis (Fernandez-Calvino et al., 2011; Simpson et al., 2009; Salmon and Bayer, 2013). In addition, the presence of phytosterols, canonical lipid components of microdomains (Mongrand et al., 2004; Laloi et al., 2007) was inferred from experiments in which treatment with a sequestering sterol compound promotes the relocalization of remorin from the detergent insoluble membranes to the detergent soluble membrane fraction and a change of its distribution from clusters (microdomain organization) to random positions (Raffaele et al., 2009). These evidences suggest the presence of microdomains in the PD and imply, but do not assure, that the general structural design of PD microdomains follows the same principles operating in known membrane domains.

In plants, as in other eukaryotes, sphingolipids, together with sterols are essential constituents of membrane microdomains (Mongrand et al., 2004; Sperling et al., 2005; Laloi et al., 2007; Carmona-Salazar et al., 2011; Cacas et al., 2012). Their chemical structure, with a highly hydrophilic polar head and a hydrophobic moiety formed by a long chain fatty acid and a sphingoid base or long chain base (LCB), makes them ideal candidates

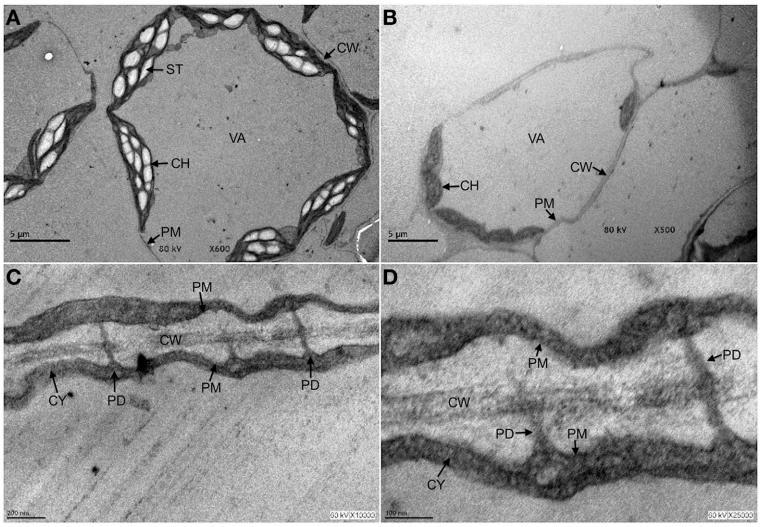

FIGURE 1 | Plasmodesmata are structures that persist under autophagy conditions. Three-week old *Arabidopsis* seedlings were exposed to 10 μM fumonisin B1 for 4 days in order to induce programmed cell death in the form of autophagy. After this time, leaf tissue was fixed and processed for transmission electron microscopy analysis as described in Saucedo-García et al. (2011a,b). **(A)** Control leaves from seedlings exposed to H2O. **(B–D)** Leaves from seedlings exposed to fumonisin B1 are shown at the indicated magnification. In **(A)**, it is observed that under control treatment, cells show a rounded shape with typically elongated chloroplasts and starch bodies, some other small organelles in the periphery and well defined plasma, vacuole and chloroplasts membranes. In **(B)**, cells from seedlings exposed to fumonisin B1 show undergoing autophagy at different stages: some cells are already empty and only the cell walls reveal their former presence; a remaining cell still displays no visible organelles, chloroplasts but with smaller size and undefined membranes. In **(C,D)**, magnifications of the FB1-treated seedlings show that cells undergoing autophagy and with few cell remnants still clearly exhibit cell walls and PD structures. CH, chloroplast; CW, cell wall; CY, cytosol; PD, plasmodesmata; PM, plasma membrane; ST, starch; VA, vacuole.

to form tightly packed regions in the membrane. These highly-ordered phases segregate in the bulk of the more extended fluid region of the membrane. Moreover, sphingolipids are very diverse due to the chemical variants of the three moieties; lipidomic analyses from *Arabidopsis thaliana* revealed about 300 molecular species of these complex lipids (Markham et al., 2006; Markham and Jaworski, 2007) Such diversity is generated by the biosynthetic enzymes and others catalyzing chemical modifications such as hydroxylation, desaturation and phosphorylation (Markham et al., 2013). T-DNA insertion mutants and gene silencing of genes coding for these enzymes have contributed to reveal the role of different sphingolipid species (**Table 1**). Plant cells require this enormous diversity in sphingolipids to carry out specific cell tasks that involve structural and signaling aspects (Chen et al., 2006; Dietrich et al., 2008; Chao et al., 2011; Saucedo-García et al., 2011a; König et al., 2012). For instance, the LCB dihydrosphingosine and a hydroxylated ceramide are involved in the programmed cell death as part of the immune response (Liang et al., 2003; Shi et al., 2007; Wang et al., 2008), the LCB sphingosine 1-P is a mediator in stomata closure in *Commelina communis* (Ng et al., 2001), but is phytosphingosine 1-P which displays this role in *Arabidopsis* (Coursol et al., 2003). This phosphorylated LCB is also produced in response to low temperatures (Cantrel et al., 2011; Dutilleul et al., 2012), while complex sphingolipids built from desaturated LCB lead to aluminum and cold tolerance in *Arabidopsis* (Ryan et al., 2007; Chen et al., 2012). A great variety of complex sphingolipids is involved in the formation of the lipid bilayer and its microdomains in the plant membranes (Sperling et al., 2005; Mongrand et al., 2010).

Arabidopsis MUTANTS, COMPLEX SPHINGOLIPIDS AND THE PD MICRODOMAIN STRUCTURE

Due to the instability (half-life 10–20 ms, Eggeling et al., 2009) and size (about 100 nm, Raffaele et al., 2009; Demir et al., 2013) microdomains are membrane zones difficult to study. Moreover, analysis of microdomains in complex structures such as the PD imposes additional problems to determine their size, distribution, and function Cacas et al., 2012. Recently developed techniques based on molecular interactions and tracking of fluorescent particles may give more information of the PD microdomains (Jacobson et al., 2007; Pike, 2009; Lingwood and Simons, 2010). Given the high elusiveness of the structure and organization of PD, microdomain studies require other approaches as the use of *Arabidopsis* mutants in genes coding enzymes of sphingolipid synthesis. This can be a very helpful strategy to dissect structural and functional features of PD microdomains and PD membranes as well.

SIGNIFICANCE OF SPHINGOLIPIDS IN PD MICRODOMAINS

It is expected that PD microdomains are mainly composed of sphingolipids and sterols as other plant PM microdomains but the factual contribution of sphingolipids to the PD structure and function is unknown. Mutants with a significantly reduced content of total sphingolipids may show low abundance or reduced size of PD and PD microdomains; this can be visualized using remorin and imaging measurements with high resolution microscopy. Some characterized *Arabidopsis* mutants that are ideal for this purpose, such as line *Atlcb2b hp/Atlcb2a,* a silenceable mutant in the serine palmitoyltransferase or SPT

Table 1 | *Arabidopsis* mutants impaired in sphingolipid metabolism.

Mutant	Modified gene, encoded protein	Gene ID	Characteristics	References
fbr11-1 (lcb1)	*LCB1*, subunit of serine palmitoyltransferase (SPT)	At4g36480	Reduced sensitivity to FB1-induced cell death	Chen et al., 2006; Shi et al., 2007
lcb2a	*LCB2a,* subunit of serine palmitoyltransferase (SPT)	At5g23670	Implicated in male gametogenesis and embryogenesis. Reduced sensitivity to FB1.	Dietrich et al., 2008; Saucedo-García et al., 2011a,b
Atlcb2b hp/Atlcb2a	*LCB2a LCB2b*, subunits of serine palmitoyltransferase (SPT)	At5g23670/ At3g48780	Inducible silencing of *Atlcb2b* in a *Atlcb2a* mutant background. Reduced total sphingolipid content upon induction with methoxyfenozide	Dietrich et al., 2008
tsc10a	*TSC10A*, 3-ketodihydrosphinganine reductase	At3g06060	Increased content of Na, K and Rb ions and decreased levels of Mg, Ca, Fe and Mo. Contributes with > 95% of the 3-KDS reductase activity in *Arabidopsis*.	Chao et al., 2011
tsc10b	*TSC10B*, 3-ketodihydrosphinganine reductase	At5g19200	Decreased content of K and Rb ions and increased Ca and Mo	Chao et al., 2011
loh1	*LOH1,* very-long-acyl-chain ceramide synthase (CS II)	At3g25540	Complete depletion of ceramides with a fatty acid acyl chain longer than C18 and excessive amounts of sphingolipids containing C16:0	Markham et al., 2011
loh2	*LOH2,* long-acyl-chain ceramide synthase (CS I)	At3g19260	Depletion of sphingolipids with fatty acids of 16 C. High sensitivity to FB1 and AAL toxin	Markham et al., 2011
loh3	*LOH3,* very-long-acyl-chain ceramide synthase (CS II)	At1g13580	Complete depletion of ceramides with fatty acid acyl chains longer than C18 and excessive amounts of sphingolipids containing 16:0	Markham et al., 2011
sld1	*SLD1,* Δ8 desaturase	At3g61580	Large reduction of Δ8 unsaturated LCB Reduction in glucosylceramide levels and increase in glycosyl inositolphosphoceramides	Chen et al., 2012
sld2	*SLD2,* Δ8 desaturase	At2g46210	Little reduction of Δ8 unsaturated LC. Reduction in glucosylceramide levels and increase in glycosyl inositolphosphoceramides	Chen et al., 2012
sld1-sld2	*SLD1,* Δ8 desaturase- SLD2 Δ8 desaturase	At3g61580/ At2g46210	Enhanced sensitivity to low temperature, grown at 0°C shows premature senescense and chlorotic lesions. Reduction in glucosylceramide levels and increase in glycosyl insitolphosphoceramides	Chen et al., 2012

(Continued)

Table 1 | Continued

Mutant	Modified gene, encoded protein	Gene ID	Characteristics	References
Δ4 des	*Δ4 DES*, Δ4 desaturase of LCB	At4g04930	Selective expression in flower and pollen. Reduced content of glucosylceramide in flowers. Channeling of substrates to the glucosylceramide synthesis	Michaelson et al., 2009
ads2	*ADS2,* acyl-CoA desaturase	At2g31360	Reduced levels of 24:1-CoA and 26:1-CoA	Smith et al., 2013
sbh1	*SBH1,* C4-hydroxylase of LCB	At1g69640	Reduced content of trihydroxy LCB	Chen et al., 2008
sbh2	*SBH2,* C4-hydroxylase of LCB	At1g14290	Reduced content of trihydroxy LCB	Chen et al., 2008
sbh1/sbh2	*SBH1,* C4-hydroxylase- SBH2, C4-hydroxylase of LCB	At1g69640/ At1g14290	Lack of trihydroxy LCB. Accumulation of total sphingolipids with predominantly C16 fatty acids. Spontaneous programmed cell death. Defects in cell elongation and cell division	Chen et al., 2008
fah1	*FAH1,* hydroxylase of FA	At2g34770	Reduced content of sphigolipids with Δ-hydroxylated fatty acids	König et al., 2012
fah2	*FAH2,* hydroxylase of FA	At4g20870	Reduced content of sphingolipids with Δ-hydroxylated fatty acids	König et al., 2012
fah1/fah2	*FAH1,* hydroxylase- FAH2, hydroxylase of FA	At2g34770/ At4g20870	Increased ceramide and salycilate levels. Reduced leaf and root growth. Enhanced resistance to biotrophic pathogens	König et al., 2012
erh1	*ERH1*, inositolphosphorylceramide synthase (IPCS)	At2g37940	Enhanced transcription of *RPW8* and RPW8-dependent spontaneous HR-like cell death in leaf tissues, and reduction in plant height. Salicylic acid accumulation	Wang et al., 2008
lcbk2	*LCBK2*, LCB kinase	At2g46090	Involved in the phosphorylation of LCB in chilling response	Dutilleul et al., 2012
sphk1	SPHK, sphingosine kinase	At4g21540	Involved in guard cell ABA signaling and seed germination	Coursol et al., 2003; Worrall et al., 2008
acd5	CERK, ceramide kinase	At5g51290	Increased content of ceramide, susceptibility to pathogen infection	Liang et al., 2003

(first enzyme of the sphingolipid synthesis), containing 64% of total sphingolipids (Dietrich et al., 2008). Other mutant lines, *tsc10a and tsc10b*, in the keto-sphinganine reductase gene (second enzyme in the sphingolipid synthesis) contain only 10% of these lipids (Chao et al., 2011). The wide difference in the total sphingolipid content between these mutants provides an opportunity to estimate the quantitative involvement of these complex lipids on the structure of the PD and PD microdomains.

IMPORTANCE OF THE HYDROXYLATED GROUPS FROM THE ACYL CHAINS

The high cohesion degree of sphingolipids and sterols that contributes to the formation of the Lo (liquid-ordered phase) characteristic of the membrane microdomains is mainly due to the harmonizing sterical shapes of sphingolipids and sterols, and the cooperative hydrophobic forces between them, and also to the hydrogen bonding at their polar regions, specially those close to

the hydrophobic tails. In this zone, the presence of charged and polar groups from the LCB, fatty acids and sterols, provide the hydrogen bonding between sterols and sphingolipids that contributes to strengthen a membrane domain. Mutants impaired in the hydroxylation of sphingolipids, such as the sphingoid base hydroxylases SBH1 and SBH2 (*sbh1-1* and *sbh2-1* mutants, respectively) (Chen et al., 2008) or fatty acid hydroxylases FAH1 and FAH2 (*fah1-fah2* mutants, respectively) (König et al., 2012) can be helpful in this matter.

IMPORTANCE OF THE DOUBLE BONDS FROM THE ACYL CHAINS

Saturated and all-trans acyl chains from fatty acids and LCB from sphingolipids favor their interaction with the planar sterol ring system producing a tight packing effect that characterizes the Lo phase of microdomains (Simons and Vaz, 2004). Mutants with different expression of desaturases of the sphingoid chain, such as lines Δ*4 des*, *sld1* and *sld2* (Sperling et al., 1998; Michaelson et al., 2009; Chen et al., 2012) or mutants in the fatty acid desaturase of sphingolipids, such as line *ads2* (Smith et al., 2013) may shed light on the relevance of the presence of saturated acyl chains to the configuration and stability of the PD microdomains.

RELEVANCE OF THE SPHINGOLIPID POLAR HEAD

Caveolae, bottle-like membrane structures are a well-characterized case of stable membrane domain. They show a neck which formation is favored by the asymmetric and dense presence of sphingolipids in the outer monolayer of the membrane. This lipid effect has been explained by the carbohydrate voluminous hydrophilic heads and by the tight packing of their acyl chain region with cholesterol, promoting and stabilizing the bending of the membrane to originate the curvature of the caveolae entrance (Dart, 2010). It is possible that the asymmetric distribution of sphingolipids recruited at the inner PM monolayer at both sites of the PD entrance could accentuate the curvature of the PM. Callose deposition at these points seems to contribute to this deformation (Maule et al., 2011) that regulates the aperture of the PD (Roberts and Oparka, 2003; Epel, 2009). Another protein involved in the callose deposition is Plasmodesmata-Callose-Binding-Protein 1 (PDCB1), which could function as a structural anchor between the cell wall and the PM components of PD (Simpson et al., 2009). Taking into account that protein-protein interactions constitute a stabilization force in microdomains, PDCB1 could participate in the recruitment of lipids forming microdomains at or near the neck (Tilsner et al., 2013). The use of sphingolipid mutants would be useful to elucidate the interactions among lipid-protein-callose. In addition, the mutant *erh1,* which contains an imbalanced content of inositolphosphoceramide species (Wang et al., 2008) or the mutant *sld1* and *sld2*, which has low amount of glucosylceramide but an increased amount of glycosyl inositolphosphoceramides (Chen et al., 2012) may help to reveal the features of the sphingolipid polar heads that are significant to form the PD and their microdomains. Thus, residence of specific proteins and lipids in these membrane regions could favor the convexity of the inner monolayer of the PM (Voeltz and Prinz, 2007; Shibata et al., 2009).

RELEVANCE OF SPHINGOLIPIDS IN THE RECRUITMENT OF PROTEINS TO MEMBRANE MICRODOMAINS INVOLVED IN DEFENSE RESPONSES

It has recently been shown that key elements in regulating the flux through the PD pore are proteins like Lysin Motif Domain-Containing Glycosylphosphatidylinositol-Anchored Protein 2 (LYM2) and Plasmodesmata-Located-Protein 5 (PDLP5) which are enriched in PD membranes. LYM2 mediates the reduction of the aperture of the PD pore in the presence of the Pathogen-Associated-Molecular-Pattern (PAMP) chitin (Faulkner et al., 2013) and PDLP5 controls the permeability during bacterial infections. In the case of PDLP5, the accumulation of the phytohormone salicylic acid elicits the over-expression of this PD protein and increases the deposition of the 1,3-glucan polymer callose, reducing the PD orifice dimension (Lee et al., 2011; Wang et al., 2013). The fact that the PDLP5 resides exclusively in the center of the PD cavity, reinforces the hypothesis that this protein is located in microdomains. Signaling mechanism controlling PD function has been suggested (Brunkard et al., 2013) but it is possible that the lipidic environment is essential for certain proteins in order to keep its position in PD. In this regard, the hypothesis is that the components of the PM could sense the accumulation of the protein in order to initiate the response that will recruit callose synthases (Wang et al., 2013). One approach to investigate the role of microdomains in determining the function of the PDLP5 would be working with mutants defective in genes linked to sphingolipid metabolism and exploring whether disruption in the lipid environment of this protein affect the closure of the pore. In this respect, the use of *Arabidopsis* mutants as *Atlcb2b hp/Atlcb2a* and *tsc10a* which contain less complex sphingolipids could help to elucidate the role of sphingolipids as regulatory structures that affect PD membrane proteins.

Arabidopsis MUTANTS, LCB AND THE PD FUNCTION IN DEFENSE RESPONSES AGAINST PATHOGENS

One of the main features of the signal transduction pathways is the fast intracellular transmission of the message triggered by the initial stimulus. In many cases, this propagation can proceed beyond, moving forward to some neighbor cells or reaching even long distances to become systemic information. PD, as universal connecting pores in plant tissues, mediate the diffusion of toxic and signaling molecules. However, this flux is selective and regulated. The size exclusion limit of PD is controlled during the developmental stage of the plant and upon pathogen infection (Angell et al., 1996; Xu et al., 2012). This is necessary to limit the flux of pathogenic molecules that might disturb neighboring cells and therefore determine disease susceptibility. However, at the same time, the intercellular communication regarding other defense molecules such as sRNAs must persist in order to establish a systemic response of resistance. In this direction, it has been demonstrated that the synthesis of siRNAs (silencing RNAs) as a response to a viral infection is a very effective systemic defense reaction in plants (Marín-González and Suárez-López, 2012; Parent et al., 2012). In addition, it has been shown that an increase in callose deposition affects the signaling mediated by miRNAs (Vatén et al., 2011) and that miRNAs are involved in defense against bacterial PAMP as well (Parent et al., 2012). It is

reasonable to expect that the control of the flow under infection conditions depends on intrinsic characteristics of the PD pore determining its size and selectivity, but also from other extrinsic ones, as concentration gradients of transit molecules as sRNAs. In fact, some of these regulatory factors originate in the chloroplast (Brunkard et al., 2013). Regarding the residence of molecules responsible of the signaling implied in the regulation of the aperture/closure of the PD pore, a significant number of receptor-like kinases has been described in PD proteomic studies (Fernandez-Calvino et al., 2011), thus suggesting that these proteins may have a crucial role in determining the changes of PD structure upon infection (Lee and Lu, 2011). In this context, it is appealing the idea of exploring the structural role of sphingolipids in the PD and PD microdomains design in relation to the modulation of the pore dimensions and the selectivity of the transported molecules, in particular, the siRNAs and miRNAs movement at short- and long-distances. Mutants that express reduced amounts of sphingolipids or that may affect the curved entrance of the tunnel as those described in the former sections would be very interesting to test. These mutants can be also used to investigate the specific lipid environment that sphingolipids provide and that may constitute an important factor determining the correct allocation of transmembrane proteins involved in structural or signaling tasks in the PD domains. This experimentation may help to understand not only the dynamics of miRNAs in systemic defense responses but the sphingolipid contribution to this aspects of plant immunity.

Besides the structural role of sphingolipids to form the PD membranes and microdomains, they can participate in signaling events. In particular, LCB, precursors of complex sphingolipids, can act as second messengers in transduction pathways. LCB are synthesized in the ER by the condensation of serine and palmitoyl-CoA, reaction that is catalyzed by the serine palmitoyltransferase (SPT), yielding keto-sphinganine, which is then reduced to form sphinganine, the simplest LCB that can be enzymatically modified with hydroxylation, phosphorylation and/or unsaturation to form a variety of sphingoid species (Chen et al., 2009). Recent evidences revealed the role of LCB as signaling molecules which are second messengers in the pathway to the programmed cell death that takes place during pathogen infection, the so called Hypersensitive Response (HR) (Peer et al., 2010; Saucedo-García et al., 2011a). In this response, programmed death is manifested only in the cells surrounding the access site of the biotroph pathogens, which lack the capacity of using cell debris as source of nutrients, leaving arrested its dissemination to more distant cells.

THE ER FROM PD AS A LOCAL LCB SOURCE FOR PROPAGATION TO ADJACENT CELLS

The fact that the ER is the site of LCB synthesis raises the possibility that in the PD, the local ER is involved in the synthesis of these second messengers with the advantage that in this strategic position, they can reach downstream targets at both contiguous cells, propagating the effect in an efficient way. This could be especially useful in the case of the dissemination of the message to program cell death, since in this case, the establishment of the HR involves the destruction of a limited and therefore controlled number of cells surrounding the access site of the pathogen. In this way, PD aperture among cells close to the pathogen ingress site would be favored by receiving the message eliciting their death to restrain the pathogen spread. In addition, it should be proposed that PD from cells located at longer distances from the pathogen entry site, and which are unexpected to be programmed for death, should maintain the PD in the closed state in order to prevent the transit of the LCB and other signaling molecules. To test this, the mutants *fbr11-1*, *lcb2a*, *loh2*, and *acd5*, defective in the response to LCB accumulation or pathogen infection are useful to elucidate the role of LCBs that come from the ER-PD. These studies will contribute to understand the role of PD in the limits for cell death or survival during the HR.

INVOLVEMENT OF THE LCB PATHWAY IN THE CONTROL OF PD OPENING

As second messengers of a transduction route leading to programmed cell death, it is possible that LCB or other pathway components have a direct effect on the proteins that regulate the changes in PD aperture. For example, these sphingolipid precursors activate the salicylic acid response (De la Torre-Hernández et al., 2010; Rivas-San Vicente et al., 2013), a central pathway for local and systemic defense systems. In addition, the C-terminus of Plasmodesmata-Located-Protein 5 (PDLP5), which is rich in cysteine residues, might function as a redox-sensor of Reactive Oxygen Species (ROS) (Wang et al., 2013). These reactive molecules have also been linked to the LCB pathway (Shi et al., 2007; Lachaud et al., 2011; Saucedo-García et al., 2011b). It can be proposed that LCB might be involved in the signaling pathway that leads to the closure of the PD mediated by PDLP5 to avoid the dissemination of bacterial effectors. This could be tested using mutants like *Atlcb2b hp/Atlcb2a, tsc10a, lcb2a-1,* and *sbh1-1, sbh1-2*, shedding light on a possible relation between signaling mediated by sphingolipids and the expression of the gene encoding PDLP5 under pathogen attack. In particular, the study of pathogen proliferation and detection of ROS using these mutants that accumulate less LCB could give information about the operation of the signaling pathway at the PD at early times of pathogen infection.

In addition to the use of mutants defective in genes coding enzymes of sphingolipid metabolism, the pharmacological approach, using inhibitors of the sphingolipid synthesis—fumonisin B1 and myriocin—or degradation, (*N,N*-dimethylsphingosine), constitute an alternative strategy to promote or arrest the accumulation of sphingoid species (Merrill et al., 1993; Shi et al., 2007; De la Torre-Hernández et al., 2010; Saucedo-García et al., 2011a). This alternative has proved to be successful to explore and substantiate the role of LCB as signaling molecules and can be very useful in structural and transduction studies linking sphingolipids to the function of PD and their membrane domains.

CONCLUSION

Given the experimental difficulties to dissect the structural organization and function of PD is necessary to approach these studies with diverse strategies. A significant number of *Arabidopsis* mutants defective in genes of sphingolipid metabolism can be

used in order to know how these lipids are involved in the assembly and function of PD membranes and their domains.

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Callose homeostasis at plasmodesmata: molecular regulators and developmental relevance

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Plasmodesmata are membrane-lined channels that are located in the plant cell wall and that physically interconnect the cytoplasm and the endoplasmic reticulum (ER) of adjacent cells. Operating as controllable gates, plasmodesmata regulate the symplastic trafficking of micro- and macromolecules, such as endogenous proteins [transcription factors (TFs)] and RNA-based signals (mRNA, siRNA, etc.), hence mediating direct cell-to-cell communication and long distance signaling. Besides this physiological role, plasmodesmata also form gateways through which viral genomes can pass, largely facilitating the pernicious spread of viral infections. Plasmodesmatal trafficking is either passive (e.g., diffusion) or active and responses both to developmental and environmental stimuli. In general, plasmodesmatal conductivity is regulated by the controlled build-up of callose at the plasmodesmatal neck, largely mediated by the antagonistic action of callose synthases (CalSs) and β-1,3-glucanases. Here, in this theory and hypothesis paper, we outline the importance of callose metabolism in PD SEL control, and highlight the main molecular factors involved. In addition, we also review other proteins that regulate symplastic PD transport, both in a developmental and stress-responsive framework, and discuss on their putative role in the modulation of PD callose turn-over. Finally, we hypothesize on the role of structural sterols in the regulation of (PD) callose deposition and outline putative mechanisms by which this regulation may occur.

Keywords: plasmodesmata, callose, β-1,3-glucanase, callose synthase, symplastic transport, sterols

INTRODUCTION—PLASMODESMATA AS INTERCELLULAR CYTOPLASMIC CONNECTIONS

In plants, cell-to-cell communication either occurs through apoplastic or symplastic ways. In apoplastic signaling, molecules residing in the extracellular matrix are actively transported into the cellular cytoplasm (via exo- and endocytosis) or act as ligands targeting canonical receptors located at the outer cell layer (cell-cell signaling). In contrast, symplastic cell-to-cell transport occurs within the continuum of interconnected cytosolic domains established by specialized membrane-protruding nano-pores. These channels are called plasmodesmata (PD) and are considered the equivalent of tunneling nanotubes (TNTs) in animal cells (Baluska et al., 2004; Kragler, 2013; Mandadi and Scholthof, 2013). Structural analysis revealed that PD are cylindrical channels, 30–50 nm in diameter, that interconnect the plasma membranes of adjacent cells and that encompass a dense rod in their center, e.g., the desmotubule (DT), that has a diameter of only 10–5 nm (Tilney et al., 1991). The DT constitutes a cylinder of compressed endoplasmic reticulum (ER) that physically bridges the ER of adjacent cells. Hence, PD-mediated cell-to-cell transport may occur through three possible pathways: (1) through the cytoplasmic space, (2) along the ER membrane of the DT and (3) through the central lumen of the DT channel (Grabski et al., 1993; Cantrill et al., 1999; Guenoune-Gelbart et al., 2008; Barton et al., 2011). The median part of the PD channel is generally expanded, whereas the orifices are often constricted (e.g., the neck regions) to form a physical bottleneck, restricting symplastic transport (Ehlers and Große Westerloh, 2013).

Biogenesis of PD occurs via two distinct pathways. Primary PD originate from remnants of the ER which are left within the developing cell wall during cytokinesis, hence forming simple, linear intercellular channels. Secondary, P. D., on the other hand, originate independently of cell division and are actively incorporated into pre-existing cell walls by a process requiring cell wall thinning and membrane insertion (Ehlers and Kollmann, 2001). As a result, secondary PD are more complex showing either simple, twinned or branched (X-, Y-, and H-shaped) configurations (Lee and Sieburth, 2010). In general, the type of PD structure is temporally and spatially regulated with young tissues commonly generating simple PDs, whereas complex PD structures arise later, during differentiation and cell expansion (Ehlers and Kollmann, 2001).

PD channels physically link the plasma membrane (PM) and ER of neighboring cells and hence form a cytosolic continuity that allows non-cell-autonomous cell-to-cell trafficking as well as long distance transport. This non-selective, passive cell-to-cell movement of molecules is driven by concentration gradient-based diffusion and only applies for molecules that do not exceed the PD size exclusion limit (SEL), e.g., typically defined as the size of the largest molecules that can readily diffuse through PD. Correspondingly, several small molecules, such as water, ions, small nucleotides, small metabolites (phytohormones) and other solutes (amino acids and sugars) are symplastically transported

through PD diffusion. In addition, PD also facilitate the selective or targeted trafficking of larger macromolecules, such as homeodomain transcription factors (TFs), protein-coding RNA molecules (e.g., mRNAs) and other proteins (Kragler, 2013) through an actively regulated process. This PD trafficking mechanism involves an interaction with the PD to change the SEL enabling a directed cell-to-cell transport of macromolecules, most presumably by the integration of intrinsic movement domains and protein-protein interactivity (Kragler, 2013). Indeed, genetic studies revealed that the intercellular trafficking capacity of SHORT ROOT (SHR), KNOTTED1 (KN1), rice thioredoxin h (RPP13-1) and in the pumpkin Heat Shock Protein 70 chaperone homologs CmHsc70-1 and -2 is affected by specific allelic mutations, suggesting the presence of a specific movement domain in each of the corresponding proteins (Ishiwatari et al., 1995; Aoki et al., 2002; Kim et al., 2005a,b,c; Bolduc et al., 2008; Gallagher and Benfey, 2009). However, no "universal" autonomous movement domain conferring symplastic movement to non-related proteins has been identified, suggesting a high protein specificity and context dependence (Gallagher and Benfey, 2005). Although the underlying regulatory mechanisms are largely unknown, both active and passive PD trafficking processes are tightly controlled and strongly depend on several physiological and developmental cues; including organ tissue and body organization, developmental stage, environmental stimuli, cellular redox status, PD complexity and the nature of the signal molecule (Itaya et al., 1998; Sivaguru et al., 2000; Kim et al., 2005a,b,c; Stonebloom et al., 2012).

In a developmental perspective, PD channels constitute an important signaling vehicle to specify cell fate and identity and to coordinate tissue-specific patterning, both in a physiological framework or in response to (a)biotic stress conditions. For example, intercellular transport of LEAFY (LFY), an endogenous TF that activates floral homeotic gene expression and hence determines flower organ development, typically occurs through PD in a non-selective, diffusion-based manner (Wu et al., 2003). Similarly, a wide range of cellular RNAs, including mRNAs and small RNAs (e.g., siRNAs and miRNAs) involved in plant development and stress signaling have been reported to move from cell to cell through PD trafficking (Carlsbecker et al., 2010; Hyun et al., 2011). In addition, cell-to-cell movement of endogenous non-cell autonomous proteins (NCAPs or trafficking proteins) involved in cell fate specification (TTG1, CPC/TRY), regulation of meristem development (KN1, STM), flowering and root cell differentiation (SHR) also occurs through, P. D., albeit in a selective, actively regulated manner (Lucas et al., 1995; Kragler et al., 1998a,b; Nakajima et al., 2001; Kim et al., 2002a,b). Importantly, besides endogenous signaling, PD channels also form physical gateways that allow intercellular trafficking of viroids and viral RNA/DNA genomes in the plant tissue (Kawakami et al., 2004; Qi et al., 2004). By the PD-targeted action of viral movement proteins (MP), viruses actively expand the PD channel aperture, largely facilitating the pernicious spread of viral infections (Wolf et al., 1989; Lucas, 2006; Epel, 2009; Niehl and Heinlein, 2011). Thus, the PD-based symplastic network in plants forms an important means of intercellular communication and trafficking, both for endogenous factors (e.g., RNAs, TFs and other proteins) as well as pathogenic intruders (e.g., viruses).

CALLOSE TURNOVER AT PD—A MAJOR MECHANISM REGULATING SYMPLASTIC CONDUCTIVITY

CALLOSE HOMEOSTASIS AT PLASMODESMATA REGULATES SEL

Symplastic movement of freely diffusing molecules strongly depends on the aperture size of the PD pores, which is quantitatively expressed by the SEL. SEL is typically defined by the largest size of the molecule that can fit through the PD aperture and is mostly expressed in terms of molecular weight (M_R), using kDa as a unit (Kempers and vanBel, 1997; Oparka and Cruz, 2000). Alternatively, SEL is occasionally expressed by the hydrodynamic or Stokes radius (R_S), which does not refer to mass, but instead reflects the physical size and shape of the transported molecule (Terry and Robards, 1987).

Although there is ongoing debate about the processes involved in SEL regulation, several studies have revealed that the deposition of callose at the PD neck, e.g., the extracellular region adjacent to the plasma membrane domains at both sides of the PD channel, is a major mechanism controlling symplastic cell-to-cell connectivity in plants (Levy et al., 2007a,b; Guseman et al., 2010; Vaten et al., 2011). Callose or β-1,3-glucan, a homo-polymer of glucose that contains some β-1,6-branches, is a polysaccharide that is exclusively found among embryophytes. In plants, callose plays a pivotal role in several biological processes, including cell plate formation (Chen et al., 2009; Thiele et al., 2009), pollen development (Li et al., 2012), vascular differentiation (Slewinski et al., 2012), epidermal patterning (Chen et al., 2009; Guseman et al., 2010), root hair development and cotton fiber elongation (Waterkeyn, 1981). In addition, targeted deposition of callose also forms an important aspect of the structural defense response of plants to various biotic and abiotic stresses, such as pathogen attack (callosic plugs or papillae), metal exposure and wounding (Sivaguru et al., 2000; Jacobs et al., 2003; Chen and Kim, 2009; Hofmann et al., 2010; Ellinger et al., 2013).

Regulation of PD conductivity through callose homeostasis is a dynamic process, physically controlling the aperture size of the symplastic channel. More specifically, controlled deposition of callose in the PD neck decreases the SEL of the trans-PD cytosolic channel, hence limiting the permeability between neighboring cells. Contrary, removal of PD callose substantially enlarges PD SEL, enabling large molecules to pass, either via active or passive trafficking. Accumulation of callose at the PD neck is tightly controlled by the antagonistic action of two types of enzymes, e.g., callose synthases (CalSs) and β-1,3-glucanases (BGs), which respectively confer synthesis and degradation of the β-1,3-glucan polymer (**Figure 1**) (Chen and Kim, 2009; Zavaliev et al., 2011).

ACCUMULATION OF PD CALLOSE THROUGH CALLOSE SYNTHASES

In higher plants, putative callose synthases have been identified based on their homology to yeast FKS (e.g., FK506 hypersensitivity); the catalytic subunit of yeast β-1,3-glucan synthase (Vögeli-Lange et al., 1988; Nasser et al., 1990; Douglas et al., 1994; Qadota et al., 1996). Biochemical evidence linking CalSs to callose synthesis and deposition was first demonstrated in barley and tobacco pollen tubes (Li et al., 2003a,b; Brownfield

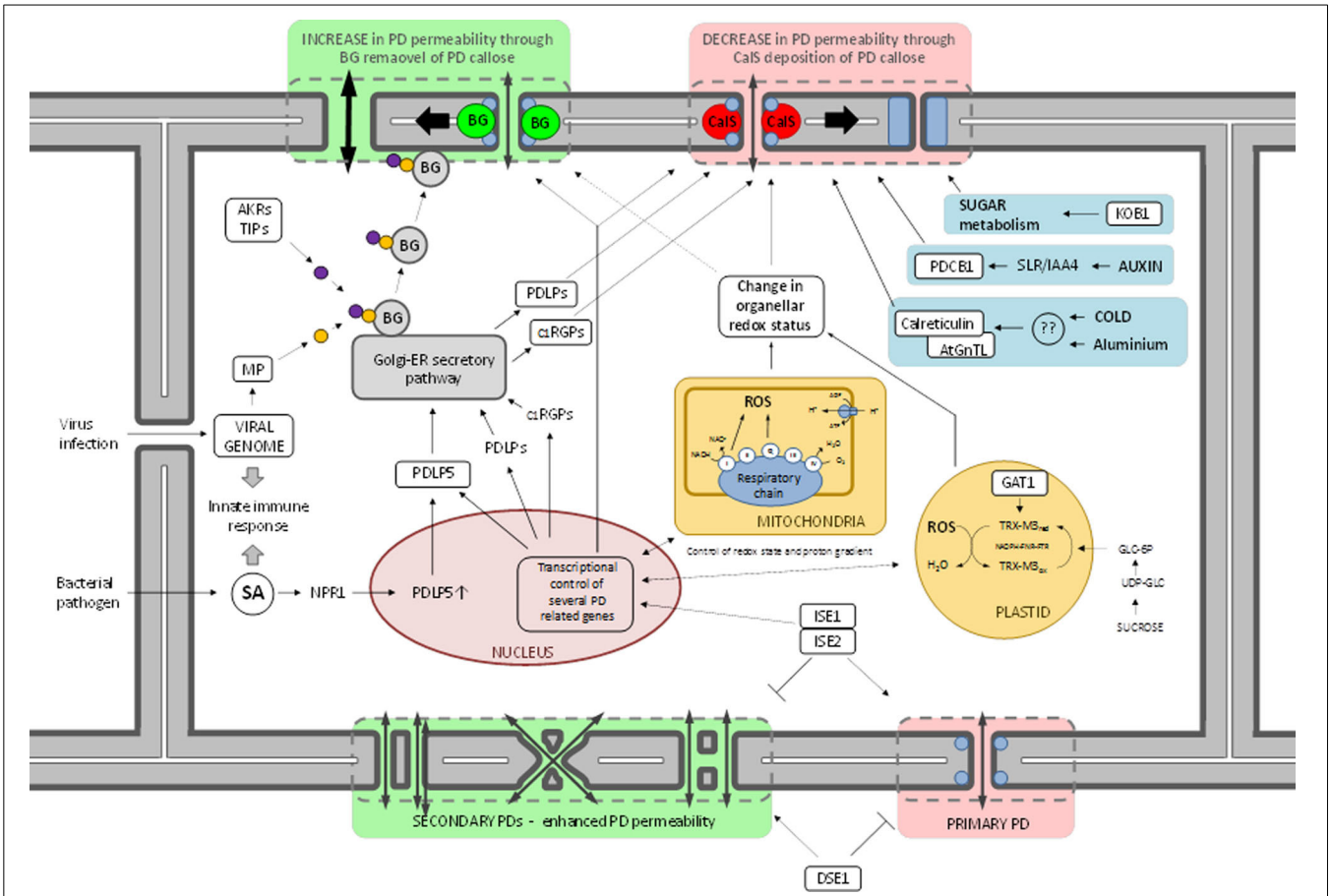

FIGURE 1 | Schematic overview representing molecular factors and signaling components involved in the regulation of PD permeability through CalS- or BG-mediated control of PD callose homeostasis.

et al., 2007) and obtained recent support by genetic studies in *Arabidopsis thaliana* (Thiele et al., 2009; Guseman et al., 2010; Vaten et al., 2011). In Arabidopsis, a total number of 12 CalSs are annotated and these are typically referred to as GLUCAN SYNTHASE-LIKE (AtGSL1-AtGSL12) proteins (Richmond and Somerville, 2000). Most *GSL* genes have 40–50 exons, except for GSL1 and GSL5 which only have two and three exons, respectively (Enns et al., 2005). In general, GSLs are large proteins (±2000 AA) that possess multiple transmembrane domain (TMD), typically clustered in two regions (e.g., N- and one C-terminal), and a large central cytoplasmic region, also termed the hydrophilic loop (Hong et al., 2001a,b; Thiele et al., 2009). The latter domain most likely contains an UDP-glucose catalytic site and a glycosyltransferase domain and acts together with the hydrophilic N-terminal region as a docking site for the interaction with various regulatory proteins, as evidenced by the presence of several glycosylation and phosphorylation sites (Verma and Hong, 2001). Consistent with their role in cell wall callose synthesis, GSLs are located in the PM and show a high substrate specificity for UDP-glucose. Nevertheless, no consensus catalytic center containing an UDP-glucose binding site has been identified yet (Brownfield et al., 2009; Zavaliev et al., 2011).

To achieve proper synthesis and deposition of callose, CalSs need to be integrated in a highly specialized protein complex; e.g., the CalS complex. Based on genetic studies using *de novo* cell plate formation, pollen tube tip growth and cotton fiber elongation, at least six proteins have been found to comprise the CalS complex, e.g., a plasma membrane-docked CalS enzyme, UDP glucose transferase1 (UGT1), phragmoplastin (Phr), Rho-like GTPase (Rop), sucrose synthase (SuSy), and annexin (ANN) (Andrawis et al., 1993; Amor et al., 1995; Shin and Brown, 1999; Hong et al., 2001a,b; Verma and Hong, 2001). SuSy (EC2.4.1.13; UDP-glucose:D-fructose 2-alpha-D-glucosyltransferase), a sugar metabolic enzyme that catalyzes the degradation of sucrose, forms an essential part of cellulose synthase complexes, more specifically in providing UDP-Glc as a primer. Similarly, CalSs also use UDP-Glc as substrate to synthesize β-1,3-glucan polymers, suggesting that supply of UDP-Glc to CalS is mediated by SuSy. Consistent with this, UGT1 is thought to transfer UDP-glucose from SuSy to CalS, hence channeling the deposition of callose to the appropriate subcellular location (Hong et al., 2001a,b). UGT1 interacts with Rop1 and this interaction only occurs in its GTP-bound state, suggesting that Rop1 regulates CalS activity through UGT1-dependent supply of substrate resources (Li et al., 1999; Verma

and Hong, 2001). A similar type of CalS regulation has been demonstrated in yeast, in which Rho forms a regulatory complex with FKS (Vögeli-Lange et al., 1988; Douglas et al., 1994; Qadota et al., 1996). Since UGT1, Rop1 and SuSy have no TMDs, the association of these proteins to CalS most likely occurs through specific interaction with the CalS hydrophilic loop site. Annexin is a membrane-bound protein with GTPase activity which is inhibited by $Ca^{2+}$and stimulated by Mg^{2+} (Shin and Brown, 1999). Despite the absence of a direct role in CalS regulation, ANN is thought to be involved in the Ca^{2+}-mediated switch from callose to cellulose synthesis; a process which also requires Mg^{2+} (Verma and Hong, 2001).

The importance of the CalS complex and its subunits for the deposition of callose at newly formed cell plates has clearly been demonstrated, however it is unclear if all these components are also required for PD callose synthesis. Phr, for example, is implicated in cell plate assembly, more specifically for squeezing exocytic vesicles into early membrane tubules to generate a transient cytokinetic tubular matrix (Gu and Verma, 1996, 1997). As this process is not required for PD SEL regulation, Phr might be dispensable for PD callose synthesis. Moreover, as Phr is not retrieved in the Arabidopsis PD proteome, whereas other CalS complex components, such as CalSs (−1, −10, and −12) and UDP-glycosyl transferases (At3g46650 and At4g14090) are (Fernandez-Calvino et al., 2011), the structural set-up of the CalS complex at PD may differ from that operating in other processes. In support of this, (Verma and Hong, 2001) suggested the existence of tissue- and process-specific differences in CalS complex composition and thereby mainly referred to the large set of CalS isozymes with varying tissue-specific expression profiles and differential Ca^{2+} requirement.

Out of the 12 callose synthases identified in Arabidopsis, only three have yet been found to have a direct role in PD callose deposition; CalS10/GSL8, CalS7/GSL7, and CalS3/GSL12. *GSL8* loss-of-function mutants (e.g., *chorus*) show a reduced accumulation of callose at the PD together with an enhanced cell-to-cell connectivity (Guseman et al., 2010). Phenotypic alterations related to defects in intercellular trafficking (stomatal clustering, excessive cell proliferation) are observed in both *gsl8* leaves and roots, indicating that GSL8 regulates PD callose deposition in a wide range of tissue types (Guseman et al., 2010; De Storme et al., 2013). This is consistent with the broad expression profile of *GSL8*, showing expression throughout all organs types (Winter et al., 2007). Besides PD callose deposition, GSL8 also plays an important role in male gametophytic development (Toller et al., 2008; Huang et al., 2009), cell wall formation (Chen et al., 2009; Thiele et al., 2009), root hair morphology (Guseman et al., 2010), plant growth (Toller et al., 2008) and reproductive ploidy stability (De Storme et al., 2013), indicating that one CalS can adopt functionality in various biological processes.

A second CalS involved in PD callose deposition is CalS7/GSL7. Mutant forms of CalS7 show a reduced accumulation of callose at the PD of incipient sieve plates and radial sieve element (SE) walls in the early stage of phloem development (perforation stage), eventually leading to SEs with fewer PD pores (Xie et al., 2011). In addition, *CalS7* loss-of-function mutants (e.g., *cs7*) are also compromised in the constituent formation of callosic plugs at phloem sieve channels. As a result, *gsl7* plants show a reduced flux of assimilates along the flowering stem, leading to a reduced stem growth and carbohydrate starvation in the terminal apex (Barratt et al., 2011). *CalS7* is only expressed in the vascular system and more specifically in the phloem SE and companion cells, indicating that CalS7-mediated callose synthesis is highly tissue-specific (Xie et al., 2011). In support of this, *gsl7* mutants do not display any other phenotypic defect, suggesting that CalS7 has no biological function other than phloem-specific PD callose synthesis (Huang et al., 2009).

A third Arabidopsis CalS with proven function in PD callose deposition is CalS3/GSL12. Using gain-of-function mutants, (Vaten et al., 2011) demonstrated that CalS3 mediates callose synthesis in the cell wall domain surrounding the, P. D., thereby regulating PD SEL and the associated cell-to-cell transport of micro- and macromolecules (e.g., SHR and miR165). CalS3 is specifically expressed in the root, seedling stele and phloem and, consistent with its biosynthetic role, shows a cell wall-associated localization pattern with foci corresponding to PD pores. Interestingly, ectopic expression of *CalS3* during phloem development partially restores the SE callose deposition in *cals7-1* loss-of-function mutants, indicating that both CalS3 and CalS7 are at least partially functionally redundant for SE-specific callose synthesis (Vaten et al., 2011). Based on this and given the various forms of PD structures connecting plant tissues, it is possible that a similar level of CalS redundancy operates in other tissue types, potentially mediated by other, yet uncharacterized, GSL family members. To tackle this, future research should include enhanced molecular-genetic studies, such as *GSL* mutant stacking and tissue-specific expression analyses.

DEGRADATION OF PD CALLOSE THROUGH β-1,3-GLUCANASES

β-1,3-glucanases or glucan endo-1,3-β-glucosidases (E.C. 3.2.1.39) are hydrolytic enzymes that catalyze the endo-type cleavage of 1,3-β-D-glucosidic linkages into single β-1,3-glucan units. These callose degrading enzymes are found in bacteria, fungi, metazoa (Bachman and McClay, 1996) and viruses (Sun et al., 2000) and are widely distributed in seed plants. Plants typically produce a diverse set of BG isoforms differing in primary structure, size, iso-electric point, cellular localization pattern and catalytic activity (Leubner-Metzger and Meins, 1999). Based on protein sequence identity, plant BGs are subdivided in three structural classes (using *Nicotiana* as a reference): (1) class I enzymes of basic proteins that localize in the vacuole (Shinshi et al., 1988), (2) class II and III isoforms of acidic proteins that are secreted in the extracellular space (Payne et al., 1990), and (3) a distinct class of intercellular "ersatz" BGs which are induced upon viral infection in class I BG-deficient *Nicotiana* mutants (Beffa et al., 1993).

In plants, BGs play a major role in the protection against the invasive action of pathogenic micro-organisms through their ability to hydrolyze β-1,3-glucan chains; an important component of the cell wall of many fungi (Kauffmann et al., 1987; Bowles, 1990; Sela-Buurlage et al., 1993; Stinzi et al., 1993; Jach et al., 1995; Douglas, 2001). In this perspective, BGs are often referred to as parthenogenesis-related 2 (PR2) family proteins. Aside from their role in pathogen defense response, plant BGs are also

implicated in many important physiological and developmental processes, including seed germination, cell division, flowering, pollen tube growth, microsporogenesis, fertilization, embryogenesis, fruit ripening, bud dormancy release and abiotic stress response (reviewed in Leubner-Metzger, 2003; Balasubramanian et al., 2012). In agreement with this diverse functionality, BG activity is transcriptionally regulated in a complex tissue- and developmental-specific manner, largely influenced by the integrated action of multiple signaling pathways, including plant hormones (ethylene, auxin, SA, and MeJA) and (a)biotic stress elicitors (e.g., toxins) (Abeles and Forrence, 1970; Vogelsang and Barz, 1993; Leubner-Metzger and Meins, 1999; Zemanek et al., 2002; Li et al., 2003a,b; Wu and Bradford, 2003). In addition, BGs may also be regulated at the post-transcriptional level, e.g., through post-transcriptional gene silencing (Decarvalho et al., 1992; de Carvalho et al., 1995).

The first study reporting on callose turn-over at PD by BG comes from (Beffa et al., 1996). Although not specifically focusing on PDs, these authors reported a reduced disease severity and a delayed spread of tobacco mosaic virus (TMV) and tobacco necrosis virus in β-1,3-glucanase-deficient *Nicotiana tabacum* and *sylvestris* plants (p35S-GLA-RNAi; TAG4.4 and SAG2.3), respectively. As this enhanced virus resistance correlated with an increased accumulation of callose at TMV-induced lesions, (Beffa et al., 1996) hypothesized that β-1,3-glucanase controls callose degradation at the, P. M., thereby influencing the cell-to-cell trafficking of viral genomes. Intercellular diffusion studies using biolistic introduction of dextrans and peptides additionally revealed that the enhanced accumulation of callose in BG-deficient TAG4.4 epidermal cells significantly reduces symplastic connectivity, indicating that BGs control the PD SEL through catalytic regulation of callose (Iglesias and Meins, 2000).

In *Arabidopsis thaliana*, the β-1,3-glucanase family contains 50 members, which are subdivided into 13 clusters based on a phylogenetic expression assay (Doxey et al., 2007; Levy et al., 2007a,b). Several of these BGs are characterized as glycosylphosphatidylinositol-anchored membrane proteins (GPI-APs), indicating for a PM-specific localization pattern (enriched in sphingolipid- and cholesterol-rich microdomains, known as lipid rafts) and a GPI-AP-like protein structure (Elortza et al., 2003). GPI-APs are typically characterized by (1) the absence of TMDs, (2) the presence of a cleavable hydrophobic N-terminal ER-directing signal peptide, and (3) a hydrophobic C-terminal tail region required for PM targeting. The latter region has a very defined and conserved structure, typically consisting of a transient TMD, a spacer and a ω site, which is recognized and processed by transamidase activity, enabling the transfer of the nascent protein to a presynthesized GPI anchor (Udenfriend and Kodukala, 1995; Hooper, 2001).

Out of the 12 Arabidopsis GPI-APs annotated as, B. G., three have been found to play a role in callose degradation at the PD, e.g., AtBG_ppap, PdBG1, and PdBG2 (Benitez-Alfonso et al., 2013). In addition, a third PdBG1/2-related protein, e.g., PdBG3, was also found to localize at the, P. D., however its presumed role in callose turnover has not been affirmed yet (Benitez-Alfonso et al., 2013). Originally identified in a screen for PD-enriched proteins, AtBG_ppap was found to localize in the ER membrane and along the cell periphery, in close association with PDs. Genetic studies revealed that loss of AtBG_ppap induces a substantial reduction in intercellular TMV trafficking together with an increased accumulating of PD callose, confirming that AtBG_papp is a PD-associated BG that promotes intercellular trafficking through degradation of callose (Levy et al., 2007a,b; Zavaliev et al., 2013). PdBG1 and its close homolog PdBG2 were identified in an Arabidopsis *in silico* screen for proteins involved in PD callose metabolism during lateral root (LR) organogenesis. PdBG1 and PdBG2 are both expressed in LR primordia and show a punctate pattern at the cell periphery, reminiscent of PD localization (Benitez-Alfonso et al., 2013). Single knock-out mutants do not show any phenotypic alteration, most likely through gene redundancy. Contrary, double *pdbg1,2* mutants show an increased accumulation of callose at the PD together with a reduced cell-to-cell macromolecular transport. In support of this, opposite effects were observed in the PdBG1-OE line. Hence, PdBG1 and 2 encode two redundantly operating BGs that negatively regulate PD callose accumulation and intercellular transport in the developing root.

DEVELOPMENTAL MODULATION OF PD CONNECTIVITY: CALLOSE TURNOVER AS A CENTRAL REGULATOR?

As multicellular sessile organisms, plants have evolved complex signaling networks to organize the spatio-temporal initiation of organ development and tissue differentiation in response both internal and external (environmental) cues. One of the major mechanisms in the regulation of this developmental plasticity is symplastic cell-to-cell communication through PD. Indeed, to transmit biological information and to impose positional programming, plant cells often use various types of mobile signals, such as hormones, RNAs, TFs, and other proteins, which are either passively or actively transported through the symplastic tract. Since the spatio-temporal spreading of these signals is essential for the regulation of morphogenesis, organ development and tissue differentiation, plants have evolved a complex dynamic regulation of PD permeability that enables symplastic continuity and restriction in a developmental framework (Han et al., 2014). To achieve this, the specific opening and closing of PD channels in plant cells is temporally and spatially controlled through a complex network of signaling molecules (**Table 1**), both in response to developmental cues and (a)biotic stress conditions.

From a mechanistic point of view, PD dynamics (opening and closing) plays an important role in organ patterning and morphogenesis as it defines the physical boundaries that separate specific groups of symplastically interconnected cells (symplastic subdomains) and hence determines the three-dimensional induction of specific cell fate and differentiation programs. More specifically, structural or functional occlusion of PD at organ boundaries in early-stage development imposes a physical barrier that restricts intercellular trafficking of specific cell fate determining proteins across the boundary, without affecting the intercellular communication and signaling within the enclosed group of cells. As a result, individual cells (for example, guard cells) or groups of cells that are physically isolated from the surrounding cells can initiate a developmental program in an synchronized and non-cell autonomous manner and develop independently, without

Table 1 | PD callose turnover enzymes and other regulators of PD callose homeostasis.

Gene	MIPS code	Protein annotation	Functional description	References
CALLOSE TURNOVER				
CalS7/GSL7	At1g06490	Callose synthase	Callose deposition in developing phloem sieve elements and in mature phloem induced by wounding	Barratt et al., 2011; Xie et al., 2011
CalS10/GSL8	At2g36850	Callose synthase	Callose deposition at cell plate, cell wall and plasmodesmata	Guseman et al., 2010
CalS3/GSL12	At5g13000	Callose synthase	Plasmodesmatal callose deposition in root and phloem tissue	Vaten et al., 2011
AtBG_ppap	At5g42100	β-1,3-glucanase	Degradation of callose at the PD neck and host factor pirated by viral genomes (MPs) to enhance virus spread by PD callose removal	Levy et al., 2007a,b; Zavaliev et al., 2013
PDBG1	At3g13560	β-1,3-glucanase	PD callose degradation in roots and leaves and role in lateral root organogenesis through regulation of PD symplastic transport	Benitez-Alfonso et al., 2013
PDBG2	At2g01630	β-1,3-glucanase		
ISE1/EMB1586	At1g12770	DEAD-box RNA helicase	Regulation of PD structure and function via transcriptional control of organellar functionality, ROS homeostasis and PD-associated proteins	Stonebloom et al., 2009; Burch-Smith and Zambryski, 2010
ISE2	At1g70070	DEVH-type RNA helicase		Kobayashi et al., 2007; Burch-Smith et al., 2011a,b
GAT1/TRX-M3	At2g15570	Thioredoxin-m3	Maintenance of plastid redox homeostasis and PD cell-to-cell connectivity in meristems, linking cytosolic ROS accumulation to enhanced PD callose deposition	Benitez-Alfonso et al., 2009
DSE1/TAN	At4g29860	WD-repeat protein	Positive regulator of PD development (e.g., from simple to complex)	Xu et al., 2012
PDLPs	Gene family (8)	PD located type I membrane receptor-like proteins	Negative regulators of PD permeability, but MP-associated host factor promoting PD movement of tubule-guided viruses	Thomas et al., 2008; Amari et al., 2010
PDLP1a	At5g43980		Negatively regulates PD permeability through unknown mechanism	Thomas et al., 2008
PDLP5/HWI1	At1g70690		Controls pathogen-induced, SA-dependent deposition of callose at PD	Lee et al., 2011; Wang et al., 2013
PDCB1	At5g61130	GPI-anchor callose binding	Positively controls PD callose accumulation, role in lat. root emergence	Simpson et al., 2009; Maule et al., 2013
PDCB2	At5g08000		Localizes to PD and binds β-1,3-glucan (callose) *in vitro*	Simpson et al., 2009
KOB1/ABI8	At3g08550	Glycosyltransferase-like protein	Putative role in cellulose synthesis, negative regulator of PD permeability	Kong et al., 2012
C1RGPs	Gene family (4)	C1 reversibly glycosylated proteins	Putative function as β-glycosyltransferase in polysaccharide synthesis	Sagi et al., 2005; Zavaliev et al., 2010
AKRs (TIP & ANK)	Gene family	AKR-repeat containing proteins	Interact with viral MPs to enhance virus spread by reducing PD callose accumulation through endogenous β-1,3-glucanase activity	Fridborg et al., 2003; Ueki et al., 2010
CRT1	At1g56340	ER-ass. Ca^{2+}-binding chaperone	Regulates Ca^{2+}homeostasis, interacts with MP and inhibits viral movement through, P. D., positively correlated to stress-induced PD callose deposition	Chen et al., 2005; Sivaguru et al., 2000
AtGnTL	At3g52060	β-1,6-N-acetylglucosaminyl transferase-like enzyme	Interacts with AtCRT1, putatively processing PD cargo proteins	Zalepa-King and Citovsky, 2013

affecting the cell fate of the surrounding cells (Kragler et al., 1998a,b; Ehlers et al., 1999).

Based on this, regulation of PD trafficking and intercellular communication is highly relevant for many plant developmental processes, including embryo body and primary root patterning (Kim and Zambryski, 2005; Kim et al., 2005a,b,c; Benitez-Alfonso et al., 2013), shoot apical meristem (SAM) determination (Gisel et al., 2002), flower organ development and stomatal cell differentiation (Guseman et al., 2010). Moreover, several biological processes have been found to depend on symplastic trafficking and spatio-temporal isolation of mobile non-cell-autonomous transcriptional regulators, such as STM and WUS in SAM maintenance and TTG and CPC in trichome patterning formation (reviewed in Han et al., 2014). However, despite their key role in organogenesis and morphogenesis, little is yet known about the molecular factors controlling tissue-specific PD functioning. In this paragraph, major proteins involved in the developmental or environmental control of PD trafficking are reviewed, with a particular focus on those proteins that regulate cell-to-cell connectivity through modulation of PD callose homeostasis.

ISEs AND DSE1 CONTROL SYMPLASTIC DOMAIN ISOLATION DURING EMBRYOGENESIS

During the last decade, embryogenesis has become one of the main model systems for studying developmental and molecular regulation of PD trafficking (Zambryski et al., 2012). During Arabidopsis embryogenesis, intercellular transport is regulated in a temporal and spatial manner, safeguarding the establishment of symplastic domains that give rise to the major organs. More specifically, early stage embryos constitute a single symplastic domain in which all cells are interconnected through functional PD channels (Kim et al., 2002a,b). Starting from the mid-torpedo stage, however, PD SEL significantly decreases, inhibiting cell-to-cell transport of large tracers (±10 kDa) but still allowing transfer of small (±0.5 kDa) molecules. This down-regulation of PD aperture correlates with the initiation of autotrophic embryo development, suggesting that symplastic domain isolation at this stage is essential for further cell expansion and the onset of autonomous developmental programming (Kim et al., 2002a,b).

The relevance of symplastic domain isolation in early embryogenesis was confirmed by the isolation of two mutants, namely *increased size exclusion limit* (*ise*) *1* and *2*, which appear embryo lethal due to a failure to downregulate cell-to-cell connectivity at the mid-torpedo stage (Kobayashi et al., 2007). Ultrastructural analysis revealed that *ise1* and *2* embryos at this stage contain higher proportions of branched and twinned PD compared to wild type, suggesting that the prolonged symplastic continuity is not caused by a reduced PD closure, but rather by an increased number of complex branched PDs (Stonebloom et al., 2009; Burch-Smith and Zambryski, 2010). Interestingly, RNA silencing of ISE1 and 2 in *N. benthamiana* sink leaves induced a *de novo* formation of secondary PDs together with an increased intercellular diffusion of GFP tracers (Burch-Smith and Zambryski, 2010), suggesting that ISE1 and ISE2 act as negative regulators of secondary PD formation and by this way control symplastic connectivity (**Figure 1**) (Burch-Smith et al., 2011a,b). However, this is inconsistent the general notion that formation of branched (complex) PDs is usually associated with a down regulation of PD SEL (Itaya et al., 1998; Oparka et al., 1999; Crawford and Zambryski, 2001). For example, GFP diffusion studies revealed that leaves which undergo sink-source transition show a dramatic restriction of non-selective PD trafficking together with an enhanced formation of complex PD channels (Oparka et al., 1999; Crawford and Zambryski, 2001; Fitzgibbon et al., 2013). Hence, these findings suggest that the increased PD permeability in *ise1* and *2* mutants is not related to changes in PD structural complexity, but rather rely on other changes in PD regulation, such as occlusion by callose or cytoskeletal dynamics. Alternatively, there may exist a high level of tissue-specificity and developmental dependence in the PD trafficking capacity of both simple and complex, P. D., as already indicated by other instances (Itaya et al., 1998).

ISE2 encodes a putative cytoplasmic DEVH-box RNA helicase that localizes to chloroplast stroma (Kobayashi et al., 2007; Burch-Smith et al., 2011a,b). Based on its role in RNA processing and post-transcriptional gene silencing, ISE2 most likely controls PD biogenesis and permeability through the regulation of RNA metabolism and gene expression. *ISE1*, on the other hand, encodes a plant-specific, but highly conserved DEAD-box RNA helicase that specifically localizes to mitochondria (Stonebloom et al., 2009). Correspondingly, *ise1* mutant embryos exhibit a disrupted mitochondrial proton gradient and an increased level of reactive oxygen species (ROS), indicating that ISE1 is implicated in the maintenance of the mitochondrial redox metabolism. Although seemingly unrelated, (Stonebloom et al., 2012) further explored the putative link between organelle redox homeostasis and PD transport and revealed that oxidative shifts in mitochondria and reductive shifts in plastids substantially enhance PD symplastic permeability, confirming the notion that the organellar redox state (e.g., in mitochondria and plastids) is an important regulator of PD cell-to-cell trafficking. However, whether this relates to the structural configuration of PD (primary or secondary) or the functional modulation of PD cell-to-cell connectivity (e.g., callose deposition) is still largely unknown. Recently, treatments with salicylic acid (SA), a biotic stress-related plant hormone, has been found to substantially accelerate the conversion of simple to complex PDs (Fitzgibbon et al., 2013). Interestingly, SA production is strongly correlated with the accumulation of ROS (e.g., oxidative burst under stress conditions) (Dat et al., 2007; Yuan and Lin, 2008), suggesting that redox related changes in intercellular permeability may be caused by modifications of the PD structure. Contrary, SA application has also been found to induce synthesis of PD callose, hence limiting cell-to-cell spread of fluorescent tracers and TMV (Krasavina et al., 2002; Wang et al., 2013), suggesting that redox-mediated PD SEL modulation may alternatively be controlled by PD callose homeostasis.

Further insights into the molecular mechanism by which the organellar redox state influences PD trafficking are provided the Arabidopsis *gfp arrested trafficking 1* (*gat1*) mutant. *Gat1* carries a mutation in *THIOREDOXIN-m3* (*TRX-m3*) and accumulates ROS (e.g., H_2O_2) through an oxidative shift in non-green plastid types (**Figure 1**) (Collin et al., 2003; Benitez-Alfonso et al., 2009). Similar as *ise1* and *2*, *gat1* generates an increased number

of secondary, branched PDs. However, contrary to *ise* mutants, *gat1* seedlings display a restricted intercellular transport from the phloem to the root meristem, similar as has been seen in *wild type* plants subjected to chemical oxidants. Interestingly, this reduced cell-to-cell connectivity consistently correlated with a substantial increase of callose at the, P. D., suggesting that redox-mediated control of PD function is directly controlled by callose homeostasis (**Figure 1**) (Benitez-Alfonso et al., 2009). The current hypothesis here is that oxidative cellular environments and related changes in metabolic activity induce the synthesis of PD callose by callose synthases, similarly as has been suggested by other instances (Benitez-Alfonso and Jackson, 2009). Indeed, several developmental and stress-induced processes suggest for a co-regulation of ROS and callose homeostasis in the control of PD permeability (Bolwell et al., 2002; Bussotti et al., 2005; Benitez-Alfonso et al., 2011). For example, exposure of plants to environmental stresses, such as heavy metals (Al, Cd, etc.) and virus infection, induces the production of ROS together with an accumulation of callose at the, P. D., leading to alterations in PD permeability (Sivaguru et al., 2000; Ueki and Citovsky, 2001; Yamamoto et al., 2002; Jones et al., 2006). Also, studies on the glutaredoxins ROXY1 and ROXY2, e.g., thioloxidoreductases that depend on the cellular redox status for their functionality (e.g., require binding of reduced glutathione, a key antioxidant compound), revealed that the expression of several callose synthase genes is dysregulated in the double *roxy1 roxy2* mutant (Xing and Zachgo, 2008). In addition, studies in castor bean revealed that a reduction in antioxidant capacity and an increased level of ROS spatially and temporally coincides with an accumulation of callose at the PD (Jongebloed et al., 2004). Moreover, ROS has been suggested to regulate the synthesis of callose during stomatal closure and as a response to (a)biotic stress cues, such as to dehydration and some fungal elicitors. Based on these and other findings, (Benitez-Alfonso et al., 2011) proposed a hypothetical model that outlines the redox regulation mechanism of symplastic transport, integrating both intercellular ROS producers (e.g., NADPH oxidases and peroxidases) and redox maintainers (GAT1, ISE1, etc.), ROS-activated Ca^{2+} influx and the associated activation of calcium-binding proteins, including the CalS complex. However, despite accumulating data that support an important role for the cellular redox state in the regulation of PD transport and callose homeostasis, underlying molecular mechanisms are as yet largely unknown (Benitez-Alfonso et al., 2011). Recent studies suggest a putative involvement of gene regulation (Burch-Smith et al., 2011a,b). Indeed, whole genome expression analysis of *ise1* and *2* showed a substantial change in gene expression, with more than 1000 genes transcriptionally altered. Interestingly, these not only include nuclear-encoded organelle genes, but also comprise genes involved in cellulose synthesis and PD regulation. The latter set includes GSL8 and AtBG_ppap and several other PD callose-related proteins, suggesting that regulation of PD transport through ISEs is directly controlled via transcriptional regulation of PD callose homeostasis (Burch-Smith et al., 2011a,b). However, although the ISE RNA helicase activity indicates for a direct transcriptional control, these findings may alternatively suggest an indirect link between redox homeostasis and PD function, implying a strong integration between redox regulation, organelle-nucleus cross-talk, and transcriptional modulation of PD callose deposition and symplastic connectivity (Burch-Smith and Zambryski, 2012).

Alternatively, ROS may also have a direct impact on the PD structure through its non-enzymatic, cell wall-loosening capacity (Ehlers and Große Westerloh, 2013). Indeed, specific ROS compounds, such as endogenous hydroxyl radicals (OH°), can cleave cell wall polymers (Schweikert et al., 2000) and modulate the cell wall structure to promote growth (Schopfer et al., 2002; Liszkay et al., 2003), suggesting that they may influence cell-to-cell transport through a structural modification of the PD channel. In support of this, two essential components required for the generation of OH, e.g., the precursor H_2O_2 and peroxidases, have been detected in the cell walls of the stem cambial zone in tomato; a region that shows a strong dynamic regulation of PD permeability(Ehlers and van Bel, 2010). In addition, class III peroxidases have been identified in the Arabidopsis PD proteome, however, their localization has not yet been demonstrated (Fernandez-Calvino et al., 2011).

Alterations in embryonic cell-to-cell transport are also observed in the *Arabidopsis decreased size exclusion limit1* (*dse1*) mutant. Opposite to *ise1* and *2*, which show a prolonged symplastic connectivity, *dse1* embryos exhibit a reduced level of cell-to-cell transport at the mid-torpedo stage (Xu et al., 2012). Correspondingly, *dse1* embryos contain a reduced fraction of branched and twinned PDs compared to wild type. *AtDSE1/EMB2757/TANMEI* encodes a WD40 protein that is highly conserved in all eukaryotes. It localizes both to the nucleus and cytoplasm and is expressed in all stages of plant development. Contrary to *DSE1* null alleles, which are embryo lethal (Yamagishi et al., 2005), the *dse1* allele (point mutation at splice donor site) causes less severe developmental defects; including reduced plant stature, delayed flower initiation, loss of apical dominance, homeotic flower defects and gametophytic sterility (Xu et al., 2012). Altogether, these findings suggest that DSE1-mediated control of symplastic permeability is not only important for embryo development, but also for other developmental processes. Correspondingly, DSE1VIGS silencing substantially reduced symplastic cell-to-cell connectivity in *N. benthamiana* leaves, indicating that DSE1 is an important regulator of PD permeability in several tissue types (Xu et al., 2012). WD40 proteins typically act as structural platforms for the establishment of protein complexes that have an important function in various pathways, including signal transduction, nuclear export, protein trafficking and pre-RNA processing (Smith et al., 1999). However, the exact mechanism by which DSE1 promotes secondary PD formation (**Figure 1**) and controls symplastic permeability is as yet unknown.

PDLPs: DEVELOPMENTAL INTEGRATORS OF SYMPLASTIC SIGNALING VIA PD CALLOSE DEPOSITION?

Symplastic domain isolation is not only important for embryo patterning and morphogenesis, but also for several other processes that occur later in development, for example for regulating SAM size and identity. Fluorescent tracer movement studies revealed that there is a distinct temporal and spatial regulation of intercellular connectivity at the apex, typically characterized by

a strong permeability in vegetative meristems which is blocked upon flowering initiation (Gisel et al., 1999, 2002). The strong correlation between flower induction and temporary restriction in cell-to-cell connectivity suggests that flowering onset is controlled by the reduced transport of a floral repressor or that floral meristem development requires spatial symplastic isolation (e.g., to physically isolate gene expression or RNA mobility), as also observed in other developmental decisions (Gisel et al., 2002). However, it is not clear whether symplastic connectivity around the SAM at flower initiation is completely blocked or still allows transfer of small molecules. Studies in Arabidopsis have revealed that the systemic floral induction signal FLOWERING LOCUS T (FT) is formed in the leaf vasculature and moves symplastically to the shoot apex to activate flower meristem identity genes, such as *APETALA1* (*AP1*) (Corbesier et al., 2007; Lin et al., 2007; Notaguchi et al., 2008; Turck et al., 2008). This non-cell-autonomous activation pathway suggests that symplastic domain isolation during flowering initiation not fully blocks intercellular signaling, but instead establishes an highly dynamic, semi-permeable organ boundary that impairs movement of specific (macro-) molecules. However, which signals are blocked and which not and the physiological relevance of this developmentally regulated permeability still remains unclear.

Little is known about the molecular regulation of symplastic connectivity in the SAM. Up till now, only two proteins related have been identified in Arabidopsis, e.g., the PD-localized (PDLP) proteins encoded by At2g33330 and At1g04520. Both proteins were originally identified in a proteomic survey of Arabidopsis suspension culture cell walls (Bayer et al., 2006) and appeared specifically expressed in subdomains of the developing SAM (Bayer et al., 2008). Similar to the other six PDLP family members, these PDLPs share features of type I membrane receptor-like proteins (e.g., two conserved Cys-rich repeats with extracellular 2xDUF26 domains) and specifically localize to the, P. D., showing a potential to regulate intercellular trafficking (Thomas et al., 2008; Lee et al., 2011). Indeed, functional characterization of one of the PDLP members, e.g., PDLP1 (At5g43980), revealed a reduced GFP cell-to-cell diffusion upon overexpression, indicating that PDLP1 negatively regulates PD trafficking. Whether this also holds true for the other PDLP members still needs to be elucidated. Interestingly, (Thomas et al., 2008) reported that single PDLP knock-out lines do not show any alteration in symplastic trafficking, suggesting a high level of functional redundancy. In support of this, *in silico* analysis revealed a high level of homology (23–78%) amongst all eight PDLP proteins. As most PDLP genes show a different spatio-temporal expression pattern, they most likely operate in a partially overlapping, tissue-specific developmental framework (Thomas et al., 2008). Bio-informatics analysis revealed that all PDLP family members share a highly conserved protein structure, consisting of an N-terminal signal peptide, a large region containing two similar domains of unknown function (DUF26), a single TMD of 21 amino acids and a short but variable C-terminal tail. Protein deletion studies demonstrated that the TMD is essential and sufficient for intercellular targeting of PDLPs to the, P. D., whereas the C-terminal region is not required for PD localization (Thomas et al., 2008). PD directed trafficking of PDLPs, as shown for PDLP1, occurs via the secretory pathway in a Brefeldin A-sensitive and COPII-dependent manner and targets PDLPs to the PM lining the interior of the PD with their short C-terminal tail in the cytoplasmic domain and their N-terminus (DUF26 domains) in the apoplast (Thomas et al., 2008; Lee et al., 2011). This configuration indicates for a function in signal perception and/or transduction. In support of this, a similar protein-membrane topology has been observed in other members of the wider group of 2xDUF26 class of proteins, including some pathogen-induced receptor-like kinases that mediate signaling from the apoplastic space to the cytoplasmic kinase module (Czernic et al., 1999; Du and Chen, 2000). Intriguingly, the specific targeting of PDLPs as receptor-like molecules at the PD hence suggest a putative role for extracellular signaling in the control of symplastic connectivity. Recently, a similar integration of apoplastic and symplastic signaling has been proposed to mediate the coordination of non-cell autonomous cell fate decisions, such as those operating in meristem maintenance and organ differentiation (Stahl and Simon, 2013).

The *PDLPs* encoded by At2g33330 and At1g04520 are strongly expressed in the SAM and localize to, P. D., albeit with a slightly different localization pattern, suggesting for a putative role in SAM symplastic domain isolation and body organization (Bayer et al., 2008). Contradictory to this, corresponding single mutants do not show any defect in shoot flower organogenesis or body structure; an observation which may also be explained by gene redundancy. In contrast, weak mutants of the GSL8 callose synthase (e.g., *et2*) exhibit clear homeotic flower defects and display alterations in shoot architecture (De Storme et al., 2013), indicating that symplastic domain isolation, e.g., through PD callose, is crucial for SAM body organization and subsequent flower organogenesis. Based on the PDLP receptor-like character and PD targeting, we hypothesize that PDLPs are important actors in the developmental control of intercellular trafficking and symplastic domain isolation, both in response to endogenous and external cues. As mode of action we presume that PDLPs positively regulate the deposition of callose at the, P. D., e.g., for example through interaction with CalS or other callose homeostasis proteins.

Initial evidence supporting this hypothesis comes from (Lee et al., 2011), who reported an inverse relationship between *PDLP5* (*HOPW1-1-INDUCED GENE 1*; At1g70690) expression level and both active (TMV) and passive (GFP and CFDA) intercellular transport, indicating that PDLP5 limits both basal PD permeability (intercellular diffusion) and actively controlled cell-to-cell movement of macromolecules, such as viral MPs. Importantly, the observed changes in intercellular connectivity appeared positively correlated with the level of PD callose, indicating that PDLP5 controls cell-to-cell permeability through modulation of PD callose accumulation. Interestingly, PDLP5 is strongly up regulated upon bacterial infection or exposure to SA (Lee et al., 2008) and thereby substantially influences the plant's susceptibility to bacterial pathogens, as demonstrated by the enhanced and reduced growth of the virulent *Pseudomonas syringae* pv *maculicola* (*Pma*—ES4326) strain upon PDLP5 loss-of-function (*pdlp5-1*) and overexpression, respectively (Lee et al., 2011). In this study, fluorescent tracer assays revealed that *Pma* infection substantially reduces PD cell-to-cell permeability through an increase in PD

callose accumulation, suggesting that PD permeability regulation through callose homeostasis constitutes an integral part of the plant innate immune response, with PDLP5 playing an important role herein. More specifically, PDLP5 is assumed to act as a PD-specific receptor molecule, integrating innate immune signals into a structural PD response. Interestingly, (Wang et al., 2013) recently reported that exogenous application of, S. A., similar as bacterial infection, suppresses cell-cell coupling through PD callose deposition and that this basal defense response requires an intact EDS1/ICS/NPR1-dependent SA biosynthesis and signaling pathway together with functional PDLP5. Interestingly, PDLP5-mediated closure of PD also depends on the associated presence and hyper accumulation of, S. A., providing strong evidence that both SA and PDLP5 act interdependently to reduce cell-to-cell coupling and symplastic connectivity. Based on this, (Wang et al., 2013) proposed a model in which PDLP5-SA crosstalk is essentially required for pathogenesis-induced restriction of cell-to-cell connectivity, e.g., via callose-based closure of PD. However, the exact mechanism and molecular factors (e.g., CalS or BG enzymes) underlying pathogen-induced accumulation of PD callose and the functional role of this process in the enhanced resistance against bacterial infections remain as yet unknown. In *Arabidopsis thaliana*, SA and pathogen infection have been found to induce expression of five of the 12 CalS enzymes, with highest up regulation for CalS1/GSL6 and CalS12/GSL5/PMR4, making them putative regulators of SA- and pathogen-induced PD callose accumulation. However, RNAi silencing of corresponding CalS enzymes did not alter callose homeostasis either at the PD or the cell plate (Jacobs et al., 2003; Nishimura et al., 2003), indicating that neither of both CalSs are implicated in PD callose deposition. It should be noted here that transcriptional upregulation of CalSs does not necessarily lead to an enhanced enzyme activity, since also other protein components (e.g., ANN, SUSY, etc.) and catalytic activators are required to form an active CalS complex (Brownfield et al., 2009). However, despite the absence of an underlying molecular mechanism, these findings collectively suggest that PDLPs are important PD-residing signal transducers that integrate both internal and external cues into a developmental response, e.g., the modulation of symplastic connectivity through PD callose accumulation.

Contrary to their role in PD closure, PDLPs may also positively regulate PD trafficking under certain conditions, e.g., more specifically in the presence of MPs produced by tubule-forming viruses. Indeed, in a recent study (Amari et al., 2010) reported that the MP of the tubule-forming Grapevine fan leaf virus (GFLV) physically interacts with all PDLP isoforms and that this interaction is essential for PD MP targeting, MP tubule assembly and GFL virus movement. Thus, contrary to their host-specific role in PD callose deposition, PDLPs are also exploited by viruses as a host-dependent mechanism to mediate PD targeting of MPs and to promote MP tubule formation, hence facilitating the pernicious spread of viral genomes.

VIRUSES COUNTER PD CALLOSE DEPOSITION BY PIRATING HOST-DEPENDENT MECHANISMS

Upon viral infection, plants induce a pathogenesis response (PR) which activates numerous defense-related pathways, such as systemic acquired resistance, PR-related gene expression, the hypersensitive reaction, hormone signaling, etc. (Mandadi and Scholthof, 2013). Interestingly, this PR response also includes an enhanced accumulation of callose around the, P. D., physically blocking the cell-to-cell spread of viral genomes (Rinne et al., 2005; Levy et al., 2007a,b; Li et al., 2012), similar as has been observed under other biotic and abiotic stress conditions (Iglesias and Meins, 2000). In response to this defense strategy, viruses have adopted a mechanism to counter this PD blockage. More specifically, viruses promote their cell-to-cell spreading in infected plant tissues by actively reducing the accumulation of callose at the PD. Several lines of evidence hereby suggest that viruses take advantage of the callose hydrolyzing activity of plant endogenous β-1,3-glucanases to directly degrade the stress-induced build-up of callose at the, P. D., hence facilitating their intercellular spread (Iglesias and Meins, 2000; Bucher et al., 2001). Interestingly, since antifungal class1 BGs are generally believed to be implicated in the constitutive and induced defense response of plants against fungal infection, this hypothesis implicates that viruses have co-opted a host cellular defense machinery against fungal infection (e.g., production of BGs) to promote their own replication and spreading (Beffa et al., 1996). However, as yet little is known about the underlying subcellular mechanism(s) and the specific host BGs or other molecular factors operating herein.

The relevance of host BGs in viral genome spreading was already demonstrated a decade ago by genetic studies in tobacco that revealed a positive relationship between the transcript level of the class I vacuolar PR-BG NtGLA and its homologs and cell-to-cell movement of TMV and other viruses (Beffa et al., 1996; Iglesias and Meins, 2000; Bucher et al., 2001; Ueki and Citovsky, 2002; Beffa and Meins, 2006). Interestingly, reduced virus spreading in BG-deficient lines hereby strongly correlated with an increased accumulation of callose at the PD, indicating that viral genomes take advantage of host BGs to reduce PD callose deposition in order to enhance intercellular spreading. Initial insights in the underlying molecular mechanism were provided by (Guenoune-Gelbart et al., 2008), who found that expression of a TMV mutant variant lacking both movement (MP) and coat protein (CP) causes an increased accumulation of PD callose in wild type *N. benthamiana* plants, whereas co-expression with ^{TMV}MP resulted in a significant reduction in PD callose accumulation. Strikingly, plants expressing only ^{TMV}MP without virus replication did not show any alteration in PD callose deposition, indicating that both replication and MP activity are required to reduce callose accumulation. To achieve this, viral MPs either target host BGs to the PD or alternatively inhibit the stress-induced synthesis of PD callose.

In support of the former mechanism, (Fridborg et al., 2003) identified three tobacco host factors (e.g., TIPs) that show concomitant interaction with the Potato virus X-encoded MP protein TGB12K and the class I vacuolar β-1,3-glucanase. Sequence analysis revealed that all three TIPs belong to the "ankyrin-repeat (AKR)" family of proteins, which are typically involved in the control of protein-protein interactions. In addition, TIP1 has been found to localize to the cytoplasm and to induce an enhanced cytoplasmic deposition at the cell periphery when co-expressed with TGB12K, suggesting that TIPs function as host

MP-BG linking proteins that mediate or enhance the transfer of β-1,3-glucanases to the PM and PD. In support of this, (Ueki et al., 2010) identified another AKR-containing protein (ANK) that interacts with MP at the PD and that positively regulates MP and TMV cell-to-cell spreading through a reduced accumulation of callose at the PD. Altogether, these data demonstrate that ANKs are host receptor proteins that are exploited by viral MPs to aid viral cell-to-cell movement by targeting β-1,3-glucanase to the PD channel. Based on these findings, (Levy et al., 2007a,b) suggested a functional model in which viral MPs mediate PD callose degradation through recruitment and PD targeting of ER-derived vesicle bodies that contain class I β-1,3-glucanases, hence dilating PD SEL and facilitating symplastic diffusion of viral genomes (reviewed in Epel, 2009). However, question remains which BGs are involved in this viral infection response.

Corresponding with a role for host BGs in virus infection, expression studies revealed that viral genomes promote host activity of specific BGs by increasing BG gene expression as part of the hypersensitive response (Vögeli-Lange et al., 1988; Nasser et al., 1990; Ward et al., 1991; Whitham et al., 2003). Whether this also comprises PD BGs remains unclear. A recent study in *Arabidopsis thaliana* revealed that several NtGLA homologs, e.g., the PR-related β-1,3-glucanases AtBG2 and 3, are significantly up regulated upon virus infection (CMV, TMV), whereas transcript levels of the PD-associated β-1,3-glucanase AtBG_ppap remain unchanged, suggesting that plasmodesmatal BGs are not included in the PR defense response nor are transcriptionally upregulated upon virus infection (Levy et al., 2007a,b; Zavaliev et al., 2013). Interestingly, upon TMV infection of *N. benthamiana*, the PR-related β-1,3-glucanase AtBG2, which normally localizes to the ER lumen, showed co-localization with ^{TMV}MP at the infection front, suggesting that not the PD-related BGs, but rather the non-PD PR-related BGs may be exploited by viruses to dilate the PD channel (Epel, 2009). In support of this, subcellular localization studies using fluorescent recombination proteins revealed that AtBG2, together with ^{TMV}MP, accumulates in TMV-induced ER bodies during early infection and that these AtBG2- and ^{TMV}MP-containing ER bodies associate with the PD at the leading edge of infection spread. However, to take part in virus-associated PD callose degradation, AtBG2 need to be translocated out of the ER to the extracellularly located callose deposits at the PD neck region and this was never observed in TMV infected tissues, neither in early or late stages of infection spread (Zavaliev et al., 2013). Moreover, transcriptional alteration of AtBG2 (e.g., overexpression and *atbg2* knock-out mutants) does not cause alterations in PD callose deposition and virus spread, indicating that even though transcription of the PR-related BG AtBG2 is induced during virus infection (Whitham et al., 2003), it is not involved in the associated promotion of symplastic connectivity (Zavaliev et al., 2013). Based on this (Zavaliev et al., 2013) suggested that the reduction in PD callose during virus infection is not mediated by activating and PD targeting of host BGs, as hypothesized earlier (Levy et al., 2007a,b; Epel, 2009), but rather by the suppression of stress-associated synthesis of callose at the PD. To test this, further research should include analysis of mutants affected in stress-induced callose synthesis and the investigation of CalS behavior (e.g., transcript level and protein stability) upon virus infection.

In addition to the MP-mediated PD callose removal by host BGs, virus-induced PD opening can also be conferred by or needs simultaneous involvement of cytoskeletal modifications at the PD. Indeed, in a recent study (Su et al., 2010) demonstrated that both CMV and TMV MP-induced increase in PD SEL requires depolymerization of actin filaments (F-actin). Moreover, severing of actin appeared to be mediated by the, M. P., at least under *in vitro* conditions. Interestingly, no link between virus-induced cytoskeletal modifications and PD callose degradation has yet been documented, suggesting that both processes act independently of each other in the process of virus-dependent regulation of PD SEL.

EPIDERMAL PATTERNING REQUIRES SYMPLASTIC ISOLATION THROUGH PD CALLOSE DEPOSITION

During plant development, restriction of symplastic signaling through PD callose homeostasis is also essential for the controlled differentiation and patterning of the epidermal cell layer. Indeed, loss of PD callose deposition (e.g., *gsl8* mutants) induces severe defects in epidermal patterning, typically characterized by stomatal cell clusters and islands of excessive cell proliferation (Chen et al., 2009; Guseman et al., 2010; De Storme et al., 2013). Formation of stomatal guard cells (GCs) is temporally and spatially controlled by a large set of activators and repressors, ensuring correct stomatal pattering and cell specification (Pillitteri and Torii, 2012). An important feature herein is the one-cell-spacing rule, which states that, across plant species, stomata always appear as single units, completely surrounded by epidermal cells (Peterson et al., 2010). This feature most likely reflects an adaptive evolution in land plants to optimize stomatal functioning, more specifically by consolidating the exchange of water and ions between GCs and adjacent cells (Pillitteri and Torii, 2012). Disruption of this one-cell-spacing rule and formation of stomatal clusters typically points toward a defect in the molecular regulation of stomatal differentiation (Geisler et al., 2000; Shpak et al., 2005; Hara et al., 2007; Wang et al., 2007), however, recent studies revealed that it may alternatively indicate for defects in PD permeability. Indeed, (Guseman et al., 2010) demonstrated that loss of PD callose deposition in GSL8-depleted Arabidopsis enables ectopic diffusion of stomatal lineage-specific TFs (e.g., SPCH, MUTE and TMM) from stomatal meristemoids to adjacent cells, hence inducing the ectopic formation of stomatal cell clusters. In addition, *gsl8* alleles also display large islands of proliferating cells (De Storme et al., 2013), suggesting that cell cycle-related determinants also show an increased cell-to-cell spreading (Chen et al., 2009). Thus, symplastic isolation of the epidermal cell layer through PD callose constitutes an important factor in the spatial confinement of several cell fate determinants, hence regulating stomatal patterning and epidermal cell proliferation.

Since the presence of stomatal clusters may indicate for defects in PD trafficking, this feature can be used as a biomarker for assessing alterations in epidermal cell-to-cell connectivity. As such, (Akita et al., 2013) retrieved stomatal clusters in sugar-treated (sucrose, glucose or fructose) Arabidopsis seedlings and demonstrated that these are caused by a reduced level of callose in stomatal meristemoids and the ectopic diffusion of stomatal

lineage-specific TFs toward adjacent cells. This finding demonstrates that sugars are involved in the regulation of symplastic communication and trafficking in the epidermis, e.g., more specifically through the modulation of PD callose. Similarly, a genetic screen based on stomatal cell clustering in Arabidopsis resulted in the identification of a novel regulator of symplastic permeability; e.g., KOBITO1 (KOB1). *Kob1-3* mutants form stomatal cell clusters (in the *erl1 erl2* background) through an ectopic intercellular trafficking of stomatal cell fate-specifying TFs, indicating that KOB1 is involved in the restriction of PD cell-to-cell coupling between stomatal cell initials and neighboring cells (Kong et al., 2012). KOB1 encodes a highly conserved, plant-specific, PM-associated glycosyl transferase-like protein that functions in the biosynthesis of cellulose during cell expansion (Pagant et al., 2002). Sequence analysis revealed that KOB1 contains a glycosyltransferase family A domain, a domain of unknown function (DUF23) and a TMD domain, showing a type II transmembrane topology typical of glycosyltransferases, with a short N-terminal region within the cytosol and a larger C-terminal tail on the extracellular side (Kong et al., 2012). Despite this functional annotation, no clear link between cellulose synthesis and PD permeability has yet been demonstrated. Indeed, (Kong et al., 2012) demonstrated that both chemical and genetic inhibition (e.g., *rsw1-1* mutant; a temperature-sensitive allele of cellulose synthase A1) of cellulose biosynthesis do not induce the formation of stomatal cell clusters in either wild type or *erl1erl2* mutant background, indicating that KOB1 is involved in a metabolic pathway that independently regulates both cellulose biogenesis and PD permeability. *Kob1-3* seedlings do not exhibit severe alterations in PD callose deposition (Kong et al., 2012), however, corresponding to the weak stomatal clustering phenotype, some minor irregularities in PD callose deposition were observed. Based on this, we hypothesize that KOB1 restricts PD trafficking in stomatal meristemoids and putatively in other tissues through regulation of callose homeostasis. In this perspective, one hypothesis could be that KOB1 is required for the targeted supply of carbohydrates to the PD CalS complex, hence regulating PD cell-to-cell connectivity (**Figure 1**). To test this hypothesis and to elucidate the precise role of KOB1 in PD trafficking restriction, further research should involve subcellular localization of KOB1 and include genetic studies to assess epistatic interactions with other PD regulating proteins, such as CalSs (e.g., GSL8) and BGs. In addition, the putative role of KOB1 in sugar-mediated control of PD SEL regulation should be explored, e.g., by assessing PD permeability and stomatal clustering in wild type and *kob1-3* mutant background upon application of sugar metabolites and signaling inhibitors.

In spite of a clear mechanistic basis, the role of the carbohydrate synthesis-related KOB1 and sugar metabolites in the regulation of PD gating suggest for the existence of a sugar-dependent signaling pathway that regulates symplastic permeability and (epidermal) cell fate specification. Interestingly, as sucrose functions as the prime substrate for the CalS complex to generate β-1,3-glucan polymers, it is plausible to assume that sugar-mediated control of PD gating is determined by CalS and its role in PD callose deposition and that KOB1 is involved in the mechanistic or regulatory control of carbohydrate supply. However, since clear data is missing, the putative mechanism by which sugars controls PD permeability remains largely elusive.

PDCBs—PUTATIVE INTEGRATORS OF PD CALLOSE STABILITY AND LATERAL ROOT ORGANOGENESIS

Similarly to PDLPs, PDCB proteins were identified in the Arabidopsis cell wall proteomics survey of (Bayer et al., 2006). All three PDCBs are members of a large family of X8 domain-containing proteins that target to the PD outer neck and that bind 1,3-β-glucan *in vitro*, hence PD callose binding proteins (PDCBs) (Simpson et al., 2009). *PDCB1, -2*, and *-3* show a widespread and overlapping expression (mainly in shoot apical region and young leaves) and neither single nor combined PDCB2 and -3 mutants display any PD-related phenotype, most likely reflecting functional redundancy with PDCB1. In contrast, PDCB1 overexpression substantially increases PD callose accumulation and reduces intercellular molecular diffusion (Simpson et al., 2009; Rutschow et al., 2011), indicating that PDCBs, or at least PDCB1, regulates PD permeability through callose homeostasis. Interestingly, PDCBs contain N- and C-terminal signal sequences that direct the protein to the external face of the PM where the mature protein is secured at the PD neck through a covalent glycosyl-phosphatidyl-inositol (GPI) linkage (Elortza et al., 2003). PDCBs are therefore suggested to function as an anchor between the PD plasma membrane and extracellularly deposited callose in the neighboring region of the cell wall (e.g., at the PD neck), hence constituting an important regulator of PD callose stability and PD SEL. Alternatively, or in addition to this function, PDCBs may also be involved in the stabilization of PD callose, e.g., by physically protecting β-1,3-glucan polymers against the degrading activity of β-1,3-glucanases. As a third alternative, (Salmon and Bayer, 2013) suggested that PDCBs may participate in the stabilization of specific microdomains, e.g., lipid rafts, at the PD cell wall (Simpson et al., 2009), hence consolidating the appropriate physicochemical cell wall configurations required for correct localization and functioning of PD- or callose homeostasis-related proteins.

Recently, several roles for PDCBs and their involvement in PD callose homeostasis in plant development have been suggested, e.g., more specifically in LR development and apical bud dormancy release (Rinne et al., 2011; Maule et al., 2013). In perennial plants, such as trees, the SAM has the capacity to switch to a dormant state in response to declining photoperiods (Bohlenius et al., 2006). This developmental transition has been found to coincide with a full structural occlusion of the PD channels in the apex, e.g., through the intra- and extracellular deposition of callose at plasmodesmatal orifices (Rinne and van der Schoot, 1998; Rinne et al., 2001; Ruonala et al., 2008), physically isolating the SAM (Rinne et al., 2011) and preventing the symplastic accessibility of flowering inducing signal conduits, such as FLOWERING LOCUS T (FT) and CENTRORADIALIS-LIKE1 (CENL1) (Mohamed et al., 2010). Release of apical bud dormancy is triggered by chilling and gibberellic acid 4 (GA4) and coincides with a full restoration of the symplastic connectivity in the meristem (Arora et al., 2003), most likely through the activity of β-1,3-glucanases (Rinne et al., 2001). In support of this, (Rinne et al., 2011) found that several putative cell wall BG genes (GH17;

glucan hydrolase family 17) in *Populus* apical meristems, together with, F. T., are significantly up regulated by chilling and GA administration. Interestingly, in the set of putative BG candidates, also a PDCB1 ortholog was identified, namely GH17_98, which contains the typical carbohydrate binding module (CBM43 or X8 domain) but lacks the GH17 family domain. Strikingly, expression analysis revealed that *GH17_98*, like all GH17s that contain a CBM43 domain (e.g., group 1a), exhibits a progressive reduction in transcript level upon chilling (Rinne et al., 2011), suggesting that shoot apical dormancy release not only requires activation of callose hydrolyzing BGs, but also depends on the removal of the callose stabilizing protein PDCB1. Based on these data, we hypothesize that PDCB1, together with BGs, is an important regulator of the removal of PD callose at SAMs during dormancy release, hence reopening symplastic signaling conduits for the movement of flowering inducing TF factors. However, to what extent PDCB1 removal is critical for this developmental transition remains unknown and needs to be addressed using genetic analyses and gene knock-out studies.

During the process of root development, the primary root forms new regions of meristem activity along its axis, e.g., the LR primordia, which subsequently emerge to form the typical branched root architecture. The programmed initiation of LR initiation and emergence is controlled by several interconnected signaling pathways, such as hormone gradient, solute flux (Himanen et al., 2002; Laplaze et al., 2007; Mishra et al., 2009; Lavenus et al., 2013) and mobile cell fate determinants (Nakajima et al., 2001; Carlsbecker et al., 2010). Recent studies hereby revealed that the spatio-temporal distribution of these cell fate signaling factors, and thus LR patterning, is coordinated by symplastic cell-to-cell communication, e.g., more specifically by the controlled deposition of callose at PD (Vaten et al., 2011; Benitez-Alfonso et al., 2013; Maule et al., 2013; Vanstraelen and Beeckman, 2013). For example, (Vaten et al., 2011) demonstrated that an increased accumulation of callose at the PD in Arabidopsis roots, e.g., through gain-of-function mutations in CalS3, substantially affects LR patterning through a reduced intercellular trafficking of major cell fate determining TFs, such as SHORT-ROOT and microRNA165. Similarly, alterations in root PD callose accumulation through transcriptional modulation of PdBG1 and/or 2 also affects LR initiation and patterning, inducing higher and lower densities of LR primordia in PdBG loss-of-function and OE lines, respectively (Benitez-Alfonso et al., 2013). Moreover, diffusion studies using fluorescent tracers (GFP and CFDA) demonstrated that LR organogenesis (initiation and emergence) in Arabidopsis is accompanied by dynamic changes in symplastic connectivity and PD callose accumulation, establishing a temporary symplastic boundary between the developing LR and the adjacent cell files during LR stage III–V (Benitez-Alfonso et al., 2013). Collectively, these data indicate that regulation of PD callose deposition and cell-to-cell connectivity is critical for determining organ identity and morphogenesis during LR development (Vanstraelen and Beeckman, 2013).

Besides the involvement of the callose homeostasis enzymes CalS3 and PdBG1 and 2, the PD callose binding protein PDCB1 has also been found to be implicated in the symplastic regulation of LR formation and patterning (Benitez-Alfonso et al., 2013; Maule et al., 2013). Indeed, transcriptional overexpression of PDCB1 has been shown to significantly increase LR density in Arabidopsis seedlings together with the ectopic induction of adjacent LR primordia. As this correlated with the ectopic formation of extended expression domains of *GATA23*, a gene that controls LR-founder cell specification in Arabidopsis (De Rybel et al., 2010), these data show that PDCB1 plays an important role in the regulation of LR initiation and patterning, most likely through its role in PD callose homeostasis and permeability. In support of this, (Maule et al., 2013) recently retrieved PDCB1 in a screen for auxin-induced proteins implicated in callose homeostasis and demonstrated that PDCB1 expression in stage III-IV LR primordia is greatly up regulated upon auxin application. Although the PDCB1 mRNA burst is relatively late compared to other LR initiation signals, loss of the PCDB1 expression response to auxin in SLR1/IAA14 gain-of-function mutants, which exhibit a reduced initiation of LRs, confirms this hypothesis and demonstrates that PDCB1 plays a functional role in auxin-regulated LR development (**Figure 1**). However, contrary to the earlier reported positive effect of PDCB1 on LR density (Benitez-Alfonso et al., 2013; Maule et al., 2013) found that PDCB1 overexpression negatively affects LR patterning, e.g., more specifically by reducing LR density and emergence rate. Interestingly, opposite effects were observed in plants exposed to the chemical 2-deoxy-D-glucose (DDG), an inhibitor of callose synthesis. Based on these findings, (Maule et al., 2013) concluded that PD callose deposition around developing LR primordia at stage III–IV and the associated restriction of symplastic connectivity is critical for the spatial demarcation of LR initiation and emergence. Moreover, based on its role as a callose stabilizing protein, PDCB1 was proposed to play an important regulatory role herein. However, question remains why LR primordia require a transient phase of symplastic restriction. One hypothesis is that LR organ emergence requires an increased accumulation of water and that symplastic isolation thereby is essential to maintain the osmotic potential and to reduce the loss of water. Alternatively, changes in PD callose deposition may alter the spreading of signals that trigger cell wall modifications, such as those required for the emergence of LRs (Maule et al., 2013).

However, despite a full characterization of the underlying regulatory mechanism, these findings collectively suggest that PDCB1, as a regulator of PD callose accumulation, operates as a developmental regulator of LR formation and root architecture, integrating auxin-dependent signaling into a symplastic domain response (e.g., symplastic restriction of LR primordia). As such, PDCB1 may constitute a major factor regulating LR initiation, emergence and patterning in response to both developmental and environmental cues (Maule et al., 2013).

C1RGPs AND CALRETICULIN-MEDIATED CONTROL OF SYMPLASTIC CONNECTIVITY IS LINKED WITH PD CALLOSE HOMEOSTASIS

During last decade, several other proteins modulating PD gating via callose have been identified, including C1RGPs, AtCRT1, and AtGnTL. The first characterized class1 reversibly glycosylated polypeptide (C1RGPs), e.g., the 41kDa SE-WAP41 protein, was identified in maize (*Zea mays*) using a proteomics survey of PD-enriched mesocotyl cell wall extracts (Epel et al., 1996).

SE-WAP41 localizes to the Golgi membrane and PD (Epel et al., 1996) and corresponding transcripts display a strong spatial and temporal correlation with primary and secondary PD formation, suggesting a putative role in PD biogenesis and/or regulation (Sagi et al., 2005). Since then, RGPs have been identified in several other plant species, including pea (Dhugga et al., 1997), Arabidopsis (Delgado et al., 1998), cotton, tomato (Selth et al., 2006), wheat and rice (Langeveld et al., 2002). The Arabidopsis genome encodes five C1RGPs (Drakakaki et al., 2006), with AtRGP2 sharing highest homology to SE-WAP41. When transiently expressed in tobacco, all Arabidopsis RGPs show PD- and Golgi-specific targeting (Sagi et al., 2005), similar as observed in pea (Dhugga et al., 1997) and maize (Epel et al., 1996). Moreover, studies using Brefeldin A demonstrated that RGPs are specifically delivered to the PD via the Golgi apparatus (Sagi et al., 2005).

Initial insights into the specific role of RGPs in PD function were provided recently. Using VIGS silencing (Burch-Smith and Zambryski, 2012) revealed that a reduced C1RGP transcript level in *N. benthamiana* enhances the spread of TMV and its P30 MP, indicating for a putative role for RGPs in PD transport regulation. In support of this, constitutive overexpression of GFP tagged *AtRGP2* in *N. tabacum* substantially reduces intercellular spread of TMV and photo-assimilates, hence yielding stunted, chlorotic plants (Zavaliev et al., 2010). Interestingly, this reduced cell-to-cell connectivity correlates with an increased accumulation of callose at the, P. D., indicating that RGPs control PD permeability most likely through modulation of PD callose (**Figure 1**). However, the underlying mechanism is as yet unknown. C1RGP family members undergo a reversible autoglycosylation in the presence of certain nucleotide UDP sugars; such as UDP-glucose, -xylose, and -galactose (Dhugga et al., 1991; Langeveld et al., 2002; Testasecca et al., 2004). Based on this and their cell wall-specific localization, C1RGPs are thought to play a role in the synthesis of cell wall polysaccharides and starch metabolism (Bocca et al., 1997; Dhugga et al., 1997; Delgado et al., 1998). More specifically, C1RGPs are presumed to act as β-glycosyltransferases (Saxena and Brown, 1999), transferring UDP sugars to putative transporters or processing complexes residing in the PM (Sagi et al., 2005). One possibility is that C1RGPs function in the delivery of UDP-sugars to CalS, hence promoting the deposition of PD callose. In support of this, C1RGPs also localize to developing cell plates during cell division (Zavaliev et al., 2010); a process that also requires CalS-dependent callose deposition (Thiele et al., 2009). Alternatively, accumulation of C1RGPs in the PM facing the PD cytoplasmic sleeve may form large homomultimeric protein complexes (∼400 kDa) (De Pino et al., 2007), physically obstructing the PD pore and hence blocking symplastic connectivity (Sagi et al., 2005; Zavaliev et al., 2010).

Calreticulin (CRT) or calregulin is an ubiquitous ER-associated Ca^{2+} binding chaperone that is implicated in various biological processes, including protein quality control, stress signaling, Ca^{2+} homeostasis, cell adhesion and ER Ca^{2+} sequestering (Opas et al., 1996; Michalak et al., 1998; Persson et al., 2001; Jia et al., 2009; Kim et al., 2013). Immuno-cytological studies in maize root apex cells revealed that calreticulin preferentially localizes to PD and accumulates at callose-enriched PDs and pit fields upon plasmolysis, indicating for a link with PD callose (Baluska et al., 1999). Interestingly, using cell wall purification studies, (Chen et al., 2005) found that calreticulin interacts with TMV MP and impairs MP targeting to the PD. Moreover, calreticulin OE was found to substantially reduce TMV cell-to-cell mobility, indicating that it negatively regulates PD permeability. In support of this, (Bilska and Sowinski, 2010) demonstrated that the decreased level of leaf assimilate export upon low temperature exposure is related to changes in PD ultrastructure, e.g., more specifically to an increase in PD calreticulin and callose. Calreticulin is therefore thought to act as a stress-responsive signaling compound that regulates PD transport through modulation of callose deposition (**Figure 1**). However, since cold-induced accumulation of calreticulin and callose are temporally separated, PD closure by calreticulin may be regulated by another mechanism, e.g., independently of callose (Bilska and Sowinski, 2010). Contrary to his hypothesis, studies in wheat and tobacco revealed a close association between aluminum (Al)-induced symplastic blockage, PD callose deposition and calreticulin expression (Sivaguru et al., 2000). Moreover, Al-induced calreticulin has been found to co-localize with PD callose deposits, supporting the notion that calreticulin regulates stress-dependent control of PD trafficking through modulation of PD callose (**Figure 1**). Based on the role of calreticulin in ER Ca^{2+} sequestering (Michalak et al., 1998; Persson et al., 2001; Wyatt et al., 2002; Christensen et al., 2008), its PD localization and the catalyzing effect of Ca^{2+} on CalS enzyme activity (Kauss, 1985; Fredrikson and Larsson, 1989; Aidemark et al., 2009), it is plausible to assume that the positive effect of calreticulin on PD closure is caused by an increased, Ca^{2+}-driven induction of CalS-mediated callose synthesis at the PD. However, as yet, the precise mechanism by which calreticulin regulates PD callose is unknown.

In search for molecular factors linking CRT and PD function, (Zalepa-King and Citovsky, 2013) identified AtGnTL, a beta-1,6-N-acetylglucosaminyl transferase-like enzyme, as an interactor of AtCRT1 that specifically targets to PD (**Figure 1**). Strikingly, loss of AtGnTL expression did not alter PD localization of TMV MP or AtCRT1, indicating that AtGnTL is not essential for PD targeting. Contrary, AtGnTL T-DNA insertional mutants show defects in seed germination and exhibit a delayed plant growth, suggesting defects in symplastic transport. Interestingly, beta-1,6-N-acetylglucosaminyl transferases are implicated in glycan synthesis, e.g., in catalyzing the attachment of oligosaccharide side chains to glycoproteins, suggesting that AtGnTL putatively regulates PD callose accumulation. Hereby, co-localization of AtGnTL with AtPDCB1 at the PD indicates for a putative involvement of PDCB1. Alternatively, based on the role of CRT1 in protein modification, (Zalepa-King and Citovsky, 2013) hypothesized that AtGnTL, either alone or together with AtCRT1, functions in the modification of PD cargo proteins during their transfer through the PD channel. In this model, PD not only confer symplastic connectivity, but also form a platform for post-translational protein modification.

A PUTATIVE ROLE FOR STEROLS IN MODULATING CALLOSE DEPOSITION AND PD PERMEABILITY

Sterols are found in all eukaryotic organism and constitute an important structural component of cell membranes, regulating

fluidity and permeability of phospholipid bilayers (Schaller, 2003). In addition, certain plant sterols, e.g., campesterol, act as a precursor of oxidized steroid hormones (brassinosteroids) that function in post-embryonic growth and development (Clouse and Sasse, 1998). Sterols are isoprenoid derivatives with a four-ring steroid nucleus synthesized from cycloartenol and converted into a wide variety of sterol variants, including cholesterol, sitosterol, campesterol and stigmasterol (Edwards and Ericsson, 1999). Several developmental alterations have been described for mutants defective in sterol biosynthesis during embryonic and post-embryonic development (Jang et al., 2000; Schrick et al., 2000; Kim et al., 2005a,b,c). These morphological changes cannot be rescued by exogenous brassinosteroid application, ascribing a regulatory function to one or more of the affected sterols (Schrick et al., 2000, 2002). Genetic and biochemical studies further demonstrated that structural sterols are also implicated in various biological processes including vascular development (Carland et al., 2002, 2010; Pullen et al., 2010), cell division and cytokinesis (Schrick et al., 2000, 2004; Hase et al., 2005; Boutte et al., 2010), fertility and ploidy stability (De Storme et al., 2013) and stomatal patterning (Qian et al., 2013). How sterols control these processes is largely unknown but may involve changes in auxin and ethylene signaling (Souter et al., 2002), auxin transport (Willemsen et al., 2003; Men et al., 2008; Pan et al., 2009; Ovecka et al., 2010), vesicle trafficking, and/or gene expression (He et al., 2003; Lindsey et al., 2003).

Functional analysis of structural sterols in plant development is hindered by the embryo lethality caused by loss-of-function of the initial steps in sterol biosynthesis. In this perspective, the non-lethality of mutant alleles of S-adenosyl-L-Met-dependent C-24 methyl transferase 2 (SMT2), an essential branching enzyme in the sterol synthesis pathway, provides an exclusive tool in the functional elucidation of structural sterols. SMT2, together with its redundantly operating ortholog SMT3, promotes the reaction that distinguishes synthesis of structural sterols from that of its BR derivates. More specifically, SMT2 catalyzes the addition of a second methyl group on the C-24 position of the steroid backbone (Husselstein et al., 1996; BouvierNave et al., 1997; Schaller et al., 1998), converting the common BR/sterol 24-methylenelophenol precursor into 24-diethylidenelophenol, hence promoting the synthesis of structural sterols. Interestingly, loss of SMT2 and/or SMT3 significantly alters the level of structural sterol without affecting the BR profile (Carland et al., 2010), indicating that these enzymes constitute an essential toolbox for the monitoring of structural sterol-dependent processes.

Single *smt2* and double *smt2smt3* mutants exhibit several developmental defects, including discontinuous cotyledon vein patterning, defective root growth, loss of apical dominance, reduced stature, sterility and homeotic flower transformations, indicating that structural sterols play an import role in patterning and organ development (Carland et al., 2002, 2010). In addition, the SMT2-defective *frill1* mutant exhibits weak defects in cell wall formation together with alterations in nuclear division and flower organ ploidy level (Hase et al., 2005; De Storme et al., 2013), indicating that structural sterols are implicated in cytokinesis, mitotic cell division and reproductive ploidy stability. Strikingly, similar developmental defects were also observed in the weak GSL8-defective *et2* mutant (De Storme et al., 2013), suggesting for the existence of a functional link between structural sterols and callose deposition. Based on this, we hypothesize that sterols are implicated in the regulation of callose deposition and homeostasis, both in *de novo* cell plate formation and cell wall assembly as well as in other callose-dependent processes. In support of this, the sterol-deficient mutants *hyd1*, *fk/hyd2*, and *smt1/cph* exhibit abnormal or ectopic accumulation of callose in their embryonic and vascular tissues (Schrick et al., 2004; Pullen et al., 2010). Moreover, several developmental alterations in sterol biosynthesis mutants suggest for a defect in callose deposition, not only at the developing cell plate, but also in other biological processes. For example, sterol-deficient seedlings typically show alterations in vascular differentiation; a process which also depends on callose synthesis, as demonstrated by the vascular patterning defects in GSL8-deficient Arabidopsis *et2* (De Storme et al., 2013) and maize *tie-dyed2* mutants (Slewinski et al., 2012). Additionally, structural sterols are also implicated in the initiation and morphogenesis of root hair growth (Souter et al., 2002; Pose et al., 2009; Ovecka et al., 2010) which requires targeted localization of callose near the tip (Kumarasinghe and Nutman, 1977; Guseman et al., 2010). The hypothesized link between sterols and callose is also supported by the excessive accumulation of callose in the mesophyll cell layer of mutant forms of the sterol ester synthesis catalyzer ERP1/PSAT1 (Phospholipid:Sterol acyl-transferase1) upon pathogen infection (Kopischke et al., 2013). Although this excessive callose build-up occurs independently of the pathogen-induced PMR4/GSL5 CalS, sterol-mediated control of callose deposition may act through another mechanism, e.g., putatively through the involvement of other CalS enzymes. Altogether, these findings suggest for a functional role for structural sterols in the regulation or stabilization of callose homeostasis in several biological processes, putatively including PD SEL regulation.

Indirect evidence supporting a functional role for sterols in plasmodesmal callose homeostasis is provided by studies in embryo and stomatal development. At first, sterol-deficiency (e.g., in the Arabidopsis *hyd1* and *hyd2/fk* mutants) typically induces defects in embryonic patterning and body organization (Jang et al., 2000; Schrick et al., 2000; Souter et al., 2002), similar as in GSL8 loss-of-function plants, suggesting for defects in symplastic domain isolation and PD regulation. Additionally, recent studies by our lab and others revealed that weak sterol biosynthesis mutants (e.g., *smt2* and *fk-J3158*) exhibit stomatal cell clusters and islands of excessive cell proliferation (Qian et al., 2013), similar as observed in the GSL8-defective *chorus* and *et2* alleles (Guseman et al., 2010; De Storme et al., 2013), supporting the notion that structural sterols control symplastic connectivity, e.g., putatively through regulation of PD callose. Opposite to this hypothesis, (Qian et al., 2013) did not link the stomatal clustering phenotype to alterations in symplastic connectivity, but instead postulated that sterols most likely control stomatal patterning through regulation of stomatal cell fate asymmetry, e.g., by a yet unknown signaling pathway. However, since the ectopic expression of cell cycle regulators and stomatal lineage-specific cell fate identifiers upon sterol alteration always appears in neighboring cells (e.g., clusters) and does not show any other spatial up regulation elsewhere (Qian et al., 2013), we believe these findings

support the hypothesis that sterols regulate cell-to-cell connectivity and symplastic permeability. The main question then is: "By what mechanism would sterols do this?" Although not the only possibility, it is conceivable that a balanced sterol composition is essentially required for proper synthesis and/or maintenance of callose at the plasmodesmatal neck. For this, both direct and indirect mechanisms can be envisaged. Direct interaction of sterols with a PD CalS would require some level of structural specificity and concentration dependency whereby upon binding a conformational change is induced that stimulates CalS enzyme activity. Since different sterol mutants show different sterol imbalances yet share the stomatal clustering phenotype (Qian et al., 2013), an indirect mechanism is more likely. In this perspective, several lines of evidence suggest for a role for structural sterols in the establishment of a specific lipid PM environment or membrane scaffolding that supports the localization and/or activity of PD CalS enzymes. In the plant's, P. M., sterols typically accumulate in highly dynamic microdomains, often referred to as detergent resistant membranes (DRM) (Borner et al., 2005; Roche et al., 2008). Biochemical analysis revealed that these DRMs contain a high fraction of key carbohydrate synthases, including CalSs, suggesting that the lipid environment in DRMs is essential for proper CalS functionality and/or localization (Bessueille et al., 2009; Srivastava et al., 2013). In a similar way, specific membrane proteins such as the auxin transporters ABCB19 and PIN1 have been found to stably associate with sterol/sphingolipid-enriched membrane fractions on which they may depend for activity (Titapiwatanakun et al., 2009). Recently, it has been suggested that PD are enriched in lipid membrane domains and that these so called "lipid rafts" play a functional role in the regulation of PD trafficking (Raffaele et al., 2009; Tilsner et al., 2011). These findings altogether suggest that the sterol composition in PD microdomains may be essential for proper localization and functionality of CalS and the associated deposition of callose. In support of this, the sterol-rich PM microdomain environment has also been suggested to be essential for correct subcellular localization, structural integrity, and/or activity of the cellulose synthase machinery; a molecular structure that closely resembles the CalS complex (Schrick et al., 2012).

Besides their role in PM integrity and scaffolding, sterol-rich microdomains are also implicated in the regulation of endocytosis and vesicle trafficking (Ikonen, 2001; Pichler and Riezman, 2004; Boutte and Grebe, 2009). Genetic studies on *de novo* cell wall formation revealed that targeting of PIN auxin transporters as well as several other PM integral and cell wall proteins to the newly formed cell plate depends on endocytosis and requires Golgi-derived vesicle trafficking (Dhonukshe et al., 2006, 2007). As localization of the Arabidopsis KNOLLE syntaxin is maintained by sterol-dependent endocytosis involving a clathrin- and DYNAMIN-RELATED PROTEIN1A-dependent mechanism (Boutte et al., 2010), a similar sterol dependency may occur for the targeting of CalS to the PD plasma membrane. In support of this, studies on *N. tabacum* pollen tube growth revealed that PM targeting of CalS occurs via endomembrane dynamics, e.g., through Golgi body and/or vesicle movement along actin filaments. In addition, (Xie et al., 2012) found that CalS5 not only localizes to the PM but also to Golgi-related endosomes, indicating that subcellular localization of CalS depends on Golgi-derived vesicle trafficking. Hence, the functional role of sterols in PD callose deposition may be related to their structural implication in PD-directed trafficking of CalS-containing endosomes. In support of this, mutants defective in sterol biosynthesis have been found to display alterations in root hair morphology (Souter et al., 2002; Pose et al., 2009; Ovecka et al., 2010). Similarly to *de novo* cell wall formation, root hair morphogenesis requires a rapid deposition of cell wall material (callose, cellulose) at the growing tip through a tight regulation of endocytosis and vesicle trafficking (Miller et al., 1997; Ryan et al., 2001; Sollner et al., 2002; Ovecka et al., 2005, 2010; Samaj et al., 2006). Loss of callose deposition in root hair tips of *smt2* sterol synthesis mutants together with the branched phenotype, similarly as observed in the GSL8 alleles *chorus* and *et2* (Guseman et al., 2010; De Storme et al., 2013), suggests that structural sterols are indeed implicated in correct endocytotic trafficking of GSL8 and PM targeting of callose deposition. Correspondingly, (Cai et al., 2011) hypothesized that a similar endocytotic mechanism is responsible for the removal of excess CalS enzyme in the subapex of tobacco pollen tubes, supporting the notion that CalS localization strongly depends on endocytotic vesicle trafficking. As such, sterols may regulate the targeting of CalSs to PD and other cell peripheral regions (PM or newly formed cell plate) through their structural involvement in endocytosis, endosome dynamics and Golgi-derived vesicle transport.

Alternatively, sterols may form a limiting substrate component in the CalS-mediated synthesis of callose. Indeed, structural membrane sterols, and more specifically sitosterol-β-glucoside (SG), constitute an important source of primer substrate for glucan polymerization (e.g., cellulose) by CesA glycosyltransferase (Peng et al., 2002; Endler and Persson, 2011). Correspondingly, CalSs may also use sitosterol as a primer for the synthesis callose polymers. Contradictory, however, (DeBolt et al., 2009) found that functional loss of both UDP-Glc:sterol glycosyl-transferases UGT80A2 and B1, e.g., enzymes that catalyze the synthesis of steryl glycosides, in *Arabidopsis* does not affect the synthesis of cellulose or any other call wall-related polysaccharide, in spite of a significant reduction of sitosterol-β-glucoside levels. Hence, these results suggest that sitosterol-β-glucoside is most likely not limiting or even dispensable for both cellulose and callose biosynthesis.

CONCLUSION

In plants, short distance cell-to-cell communication and symplastic domain isolation through structural modulation of PD constitute important processes that regulate organ morphogenesis and body patterning in response to developmental and environmental cues. During the last decades, genetic and biochemical studies have revealed that PD callose homeostasis constitutes a major mechanism regulating PD SEL and cell-to-cell trafficking, both in the framework of endogenous signaling as well as in symplastic virus spread. Recent work in Arabidopsis has led to the identification of several callose synthases and β-1,3-glucanases that play a key role in the regulation of PD callose deposition. In addition to these central players, various signaling components and effector proteins (e.g., PDLPs, PDCBs, etc.) have been

found to coordinate PD permeability in response to external cues. However, in spite of a clear role in PD callose accumulation, the molecular mechanism(s) linking the identified actors to callose homeostasis remains elusive. Hence, to gain more insight into the regulatory network determining the developmental regulation of PD callose deposition, future research should include a thorough examination of PD callose enzymes and their putative interactors (e.g., involvement of CalS complex components?) together with an advanced study of their transcriptional and (post-)translational regulation (activation, localization, etc.) by yet identified signaling components. Moreover, as PD trafficking is a tightly regulated in a temporal and spatial manner both in response to internal and external cues, current knowledge on PD regulation and callose most likely only represents a mere reflection of the whole regulatory network involved. We therefore believe that future work will contribute significantly to a better understanding of PD cell-to-cell communication, both in respect to its developmental regulation, molecular control and PD callose homeostasis.

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A phylogenetic approach to study the origin and evolution of plasmodesmata-localized glycosyl hydrolases family 17

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Colonization of the land by plants required major modifications in cellular structural composition and metabolism. Intercellular communication through plasmodesmata (PD) plays a critical role in the coordination of growth and cell activities. Changes in the form, regulation or function of these channels are likely linked to plant adaptation to the terrestrial environments. Constriction of PD aperture by deposition of callose is the best-studied mechanism in PD regulation. Glycosyl hydrolases family 17 (GHL17) are callose degrading enzymes. In Arabidopsis this is a large protein family, few of which have been PD-localized. The objective here is to identify correlations between evolution of this protein family and their role at PD and to use this information as a tool to predict the localization of candidates isolated in a proteomic screen. With this aim, we studied phylogenetic relationship between Arabidopsis GHL17 sequences and those isolated from fungi, green algae, mosses and monocot representatives. Three distinct phylogenetic clades were identified. Clade alpha contained only embryophytes sequences suggesting that this subgroup appeared during land colonization in organisms with functional PD. Accordingly, all PD-associated GHL17 proteins identified so far in *Arabidopsis thaliana* and Populus are grouped in this 'embryophytes only' phylogenetic clade. Next, we tested the use of this knowledge to discriminate between candidates isolated in the PD proteome. Transient and stable expression of GFP protein fusions confirmed PD localization for candidates contained in clade alpha but not for candidates contained in clade beta. Our results suggest that GHL17 membrane proteins contained in the alpha clade evolved and expanded during land colonization to play new roles, among others, in PD regulation.

Keywords: plasmodesmata, callose regulation, GH17 domain, beta 1,3 glucanases, phylogenetic analysis

INTRODUCTION

Cell-to-cell communication is a requisite for the evolution of multicellular organisms. Plant intercellular connections (plasmodesmata, PD) are thought to originate with the appearance of multicellularity in green algae but their structural complexity increased, presumably, as a result of changes in cell-wall composition during adaptation to terrestrial environments (Lucas and Lee, 2004; Popper et al., 2011). Similarities between intercellular connections in charophytic algae and in early land plants suggest that they have a common evolutionary origin. Plasmodesmata occur in all embryophytes (including mosses) and, in their simplest form, also appear in representatives of charophytic green algae (Franceschi et al., 1994; Cook et al., 1997; Raven, 1997; Graham et al., 2000; Qiu, 2008). The presence of phragmoplast (p, enlarged cytoplasmic connection formed in the later stages of plant cell mitosis) in the zygnematalean taxa suggest that PD likely originate during the evolution of phragmoplast-containing charophyceans (**Figure 1**).

In their primary form, PD arise during cytokinesis, presumably via enclosure of endoplasmic reticulum by cell wall depositions (Hepler, 1981; Cook et al., 1997). Important features of plant PDs (such as neck constriction and central desmotubule like structure) appear in *Chara* species but since the colonization of land by plants (more than 400 million years ago) numerous modifications in PD ultrastructure and regulation are expected. A more complete understanding of the evolutionary steps involved in the origin of plant PDs, their function and regulation should be possible through the identification of plasmodesma-associated proteins and analysis of their evolutionary appearance in charophycean algae and land plants. Plasmodesma-associated proteins have been isolated in model plants, such as Arabidopsis and tobacco, using genetic and proteomic screens but the composition of the channel in model and non-model organisms is far from being resolved (Faulkner and Maule, 2011). Genome sequencing projects and prediction tools for protein structure and targeting has been proven useful to establish protein localization and function in different intracellular compartments (e.g., Pires and Dolan, 2010; Ma et al., 2011; Tardif et al., 2012). Known PD proteins display characteristic features of membrane-localized proteins (such as secretory signal peptides, glycosyl phosphatidylinositol anchors or transmembrane domains) but no specific sequence signature for PD-binding has been yet discovered.

Recently we have obtained information on the identity of Arabidopsis PD proteins, including several callose (beta 1,3 glucans) metabolic enzymes (Levy et al., 2007; Fernandez-Calvino et al., 2011; Vaten et al., 2011; Benitez-Alfonso et al., 2013). Callose deposition at PD neck region correlates with a reduction

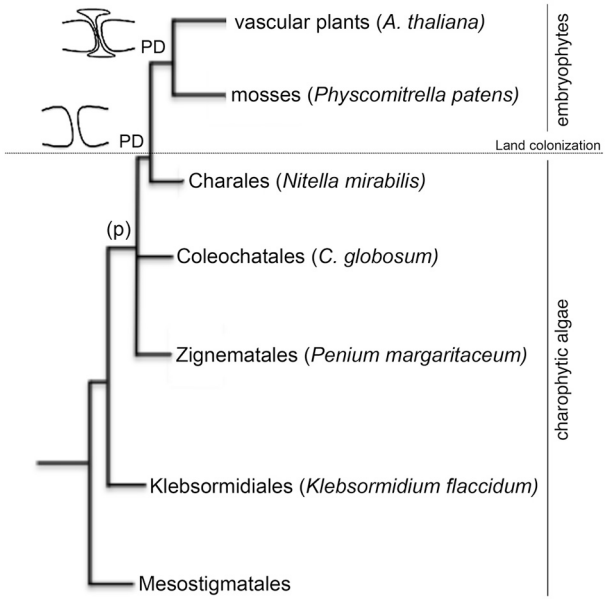

FIGURE 1 | Phylogenetic relationships of the species used in this study. The cladogram is based on the current view of land plant evolution (Qiu, 2008). Members of the order Mesostigmatales, Klebsormidiales, Zygnematales, Coleochatales, and Charales form the charophytic green algae lineage (land plant ancestors). Representatives from these orders selected for this study are named in the figure. Embryophytes (such as the moss *Physcomitrella patents* and the vascular plant *Arabidopsis thaliana*) evolved from charophytic algae during land colonization. Phragmoplast (p) were found in organisms belonging to the Coleochatales and the Charales. Plasmodesmata (PD) appeared in all embryophytes.

in symplastic transport during tissue maturation (Burch-Smith and Zambryski, 2012; Slewinski et al., 2012). Callose also acts as a reversible regulator of intercellular transport in response to developmental and environmental signals (Levy et al., 2007; Benitez-Alfonso et al., 2010; Maule et al., 2011, 2013; Rinne et al., 2011; Zavaliev et al., 2011). This implies that the activity of callose biosynthetic (callose synthases, CalS) and degrading enzymes (glycosyl hydrolase family 17, GHL17) must be rapidly and efficiently regulated at PD sites. Not surprisingly, PD-associated CalS and GHL17 proteins have been recently identified (Guseman et al., 2010; Vaten et al., 2011; Slewinski et al., 2012; Benitez-Alfonso et al., 2013; Zavaliev et al., 2013).

The role of plasmodesmata-localized GHL17 proteins in plant development and response to viral pathogens has been well established (Levy et al., 2007; Zavaliev et al., 2011; Burch-Smith and Zambryski, 2012). The identification of these enzymes in crop species could lead to the development of biotechnological approaches to improve plant growth and response to environmental and developmental signals. This task is hindered by the lack of tools to discriminate between plasma membrane (PM) and PD GHL17 proteins. Generation of fluorescent fusions and transgenics to determine intracellular localization will be required but, without any preliminary method to screen for candidates, this process could become very expensive and time consuming especially when dealing with large multigenic families such as GHL17.

Callose metabolic enzymes are conserved in fungi, oomycetes, algae and plants which indicate that this is a very ancient metabolic pathway (Bachman and McClay, 1996; Popper et al., 2011). What is not known is when this pathway was recruited to play an active role in PD regulation. The answer to this question might underlie in the evolutionary diversification of these enzymes to play PD-specific functions in land plants.

In this paper we present evidences supporting a potential correlation between the evolutionary origin of GHL17 proteins and their likelihood to target PD sites. Through phylogenetic analysis we identified a clade of membrane proteins that appear to have diverged early during land plants adaptation to terrestrial environments. The intracellular localization of predicted membrane GHL17 proteins isolated from Arabidopsis and Populus suggest that this "embryophytes only" subgroup is enriched in PD proteins (Pechanova et al., 2010; Fernandez-Calvino et al., 2011; Rinne et al., 2011; Benitez-Alfonso et al., 2013; Zavaliev et al., 2013). We used this information for the preliminary screen of 4 candidates identified through the proteomic screen of PD-enriched cell wall fractions. Two of the proteins belonged to clade alpha and were previously described to localize at PD. We tested the localization of two proteins that belonged to clade beta and found, through fluorescent imaging of m-Citrine protein fusions, that they accumulate preferentially in the apoplast. Our results suggest that at least a portion of GHL17 membrane proteins contained in clade alpha evolved in embryophytes differently from proteins contained in clade beta to specifically target PD and control callose on site.

MATERIALS AND METHODS

RETRIEVAL OF GHL17 SEQUENCES AND ANALYSIS OF PROTEIN DOMAINS

To isolate sequences containing the 1,3-beta glucosidase domain (GH17) from charophycean algae, *Physcomitrella patens* and selected embryophytes (*Arabidopsis thaliana*, *Populus trichocarpa* and *Oryza sativa*) BLAST (Altschul et al., 1990) searches were performed using as query five representative GHL17 sequences from *Arabidopsis thaliana* (At3g13560, At3g57260, At4g14080, At4g31140, At5g42100). For charophycean algae we searched the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) non-redundant (NR), high-throughput genome sequence (HTGS), whole genome shotgun (WGS), genome survey sequence (GSS) and expressed sequence tag (EST) databases. We obtained partial ESTs that were translated to amino acid sequences using Expasy translate tool. Presence of GH17 domain was confirmed in these sequences using the Conserved Domain (Marchler-Bauer et al., 2007) and SMART (http://smart.embl-heidelberg.de; Letunic et al., 2012) search engines. To isolate GH17 proteins from embryophytes sequenced genomes (*Physcomitrella patens*, *Populus trichocarpa* and *Oryza sativa*) a BLAST search against the Refseq protein database for each specific organism was performed using as query the same five Arabidopsis representative listed above and the GHL17 consensus domain sequence (cl18348). Similarly, to isolate beta-1,3-glucanases from fungi representatives (*Candida albicans, Aspergillus clavatus, Aspergillus fumigatus, Aspergillus niger, Candida glabrata, Debaryomyces hansenii, Ashbya gossypii,*

Fusarium graminearum, Kluyveromyces lactis, Saccharomyces cerevisiae, Scheffersomyces stipitis, Schizosaccharomyces pombe, Yarrowia lipolytica) the consensus domain sequence (ci18819) was used to search the reference genome databases. Only protein sequences containing GH17 domain (confirmed in SMART) and predicted to be complete were considered. Aramemnon (http://aramemnon.uni-koeln.de/request.ep) was also used to search and/or confirm the identity of the proteins isolated in the Rice annotation project database or in Phytozome.

To eliminate redundancies, and/or to identify overlapping regions in isolated ESTs, sequences obtained for each organism were aligned using Muscle (Edgar, 2004). The resulting sequences are summarized in **Table 1**. These were screened for characteristic features of this family, the presence of a secretory signal peptide (SP), glycosyl phosphatidylinositol anchor (GPI) and carbohydrate-binding module (X8), using the prediction programs SMART, SignalP 4.1 Serve, Phobius, GPI-SOM, FragAnchor, PredGPI and BIG-PI respectively (Eisenhaber et al., 2003; Fankhauser and Maser, 2005; Poisson et al., 2007; Pierleoni et al., 2008; Petersen et al., 2011; Letunic et al., 2012). According to the results obtained full length sequences were classified in the following types: type 0 showed no obvious SP (non-secreted proteins); type 1 contains SP and might (or might not) contain one or more X8 domains (predicted secreted proteins); type 2 contains SP, one or more X8 domains and GPI anchor and type 3 contains SP and GPI anchor but not X8 domain. The presence of GPI anchor in type 2 and 3 proteins was used to predict their membrane localization. The classification of the sequences analyzed is provided in **Table 2**.

ALIGNMENTS, SEQUENCE CONSERVATION, AND PHYLOGENETIC ANALYSIS

All sequences isolated from representatives of charophycean algae and fungi, *P. patens, Oryza sativa* and *Arabidopsis thaliana* (**Table 1**) were aligned using Muscle (Edgar, 2004). Sequences from algae were incomplete which generate large gaps. These gaps were mostly avoided when only the domain was used. Therefore we constructed trees with both, full sequences and domain only. These alignments are provided in Supplementary data 1. To calculate the best fitting model of amino acid evolution MEGA5 was used (Tamura et al., 2013). This suggests WAG+G+F as the best model under the Akaike Information Criterion. Dendograms were obtained using three different methods of tree reconstruction [maximum likelihood (ML), neighbor-joining (NJ) and Bayesian inference (Bayesian)]. A majority-rule consensus tree was built by Bayesian inference using Mr. Bayes (Huelsenbeck and Ronquist, 2001). Convergence was reached after 960000 generations (3720000 when using domain only) and posterior probabilities were calculated for each clade. Using the same model a ML analysis was performed with MEGA5 (Tamura et al., 2013) and bootstrap values were determined from a population of 100 replicates. A NJ tree was also generated using Phylip (Felsenstein, 1997) as well as bootstrap values, which were determined from a population of 100 replicates. The tree was visualized using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). A similar protocol was followed for phylogenetic comparison of *Arabidopsis thaliana* and *Populus trichocarpa* sequences

Table 1 | List of sequences used for constructing the phylogenetic trees.

Organism	Identifier in this paper	Sequence identifier
Klebsormidium flaccidum	KfGHL17_1	HO446722 + HO446665*
Klebsormidium flaccidum	KfGHL17_2	HO451810.1
Penium margaritaceum	PmGHL17_1	JO220251.1
Chaetosphaeridium globosum	CgGHL17_1	HO400516.1
Nitella mirabilis	NtGHL17_1	JV792233.1
Nitella mirabilis	NtGHL17_2	JV742253.1
Nitella mirabilis	NtGHL17_3	JV760383.1
Physcomitrella patens	PpGHL17_1	XP_001761806.1
Physcomitrella patens	PpGHL17_2	XP_001772420.1
Physcomitrella patens	PpGHL17_3	XP_001780679.1
Physcomitrella patens	PpGHL17_4	XP_001762206.1
Physcomitrella patens	PpGHL17_5	XP_001780506.1
Physcomitrella patens	PpGHL17_6	XP_001779924.1
Physcomitrella patens	PpGHL17_7	XP_001767901.1
Physcomitrella patens	PpGHL17_8	XP_001771454.1
Physcomitrella patens	PpGHL17_9	XP_001782572.1
Physcomitrella patens	PpGHL17_10	XP_001773368.1
Physcomitrella patens	PpGHL17_11	XP_001782548.1
Physcomitrella patens	PpGHL17_12	XP_001772976.1
Physcomitrella patens	PpGHL17_13	XP_001757439.1
Physcomitrella patens	PpGHL17_14	XP_001754617.1
Physcomitrella patens	PpGHL17_15	XP_001775842.1
Physcomitrella patens	PpGHL17_16	XP_001762304.1
Physcomitrella patens	PpGHL17_17	XP_001757144
Physcomitrella patens	PpGHL17_18	XP_001777261.1
Candida albicans	CaGHL17_1	P43070.1
Aspergillus clavatus	AcGHL17_1	XP_001269132.1
Aspergillus fumigatus	AfgHL17_1	XP_752511.1
Aspergillus niger	AnGHL17_1	XP_001392475.1
Candida glabrata	CglGHL17_1	XP_446374.1
Debaryomyces hansenii	DhGHL17_1	XP_462355.1
Ashbya gossypii	AgGHL17_1	NP_986324.2
Fusarium graminearum	FgGHL17_1	XP_383705.1
Kluyveromyces lactis	KlGHL17_1	XP_455217.1
Saccharomyces cerevisiae	ScGHL17_1	NP_011798.1
Scheffersomyces stipitis	SsGHL17_1	XP_001387556.1
Schizosaccharomyces pombe	SpoGHL17_1	NP_594455.1
Yarrowia lipolytica	YlGHL17_1	XP_500465.1
Oryza sativa	OsGHL17_1	NP_001052739.1
Oryza sativa	OsGHL17_2	NP_001044874.1
Oryza sativa	OsGHL17_3	NP_001047027.1
Oryza sativa	OsGHL17_4	NP_001046220.1
Oryza sativa	OsGHL17_5	NP_001058028.1
Oryza sativa	OsGHL17_6	NP_001044198.1

(Continued)

Table 1 | Continued

Organism	Identifier in this paper	Sequence identifier
Oryza sativa	OsGHL17_7	NP_001051111.1
Oryza sativa	OsGHL17_8	NP_001049413.1
Oryza sativa	OsGHL17_9	NP_001060087.2
Oryza sativa	OsGHL17_10	NP_001059752.1
Oryza sativa	OsGHL17_11	NP_001068140.2
Oryza sativa	OsGHL17_12	NP_001057968.1
Oryza sativa	OsGHL17_13	BAD31779.1
Oryza sativa	OsGHL17_14	NP_001173461.1
Oryza sativa	OsGHL17_15	NP_001050810.1
Oryza sativa	OsGHL17_16	NP_001056153.1
Oryza sativa	OsGHL17_17	NP_001062739.1
Oryza sativa	OsGHL17_18	BAD01673.1
Oryza sativa	OsGHL17_19	NP_001061277.1
Oryza sativa	OsGHL17_20	NP_001045844.1
Oryza sativa	OsGHL17_21	AA037977
Oryza sativa	OsGHL17_22	AAP44659
Oryza sativa	OsGHL17_23	ABF94756.1
Oryza sativa	OsGHL17_24	ABF95444.1
Arabidopsis thaliana	At2g05790	NP_178637.2
Arabidopsis thaliana	At4g26830	NP_194413.2
Arabidopsis thaliana	At5g55180	NP_001154780.1
Arabidopsis thaliana	At4g18340	NP_193568.2
Arabidopsis thaliana	At1g30080	NP_174300.2
Arabidopsis thaliana	At2g26600	NP_850082.1
Arabidopsis thaliana	At3g15800	NP_188201.1
Arabidopsis thaliana	At2g27500	NP_001031432
Arabidopsis thaliana	At5g42100	NP_974868.1
Arabidopsis thaliana	At1g32860	NP_174563.2
Arabidopsis thaliana	At5g24318	NP_001119271.1
Arabidopsis thaliana	At3g46570	NP_190241.1
Arabidopsis thaliana	At2g39640	NP_181494.1
Arabidopsis thaliana	At3g55430	NP_191103.1
Arabidopsis thaliana	At5g42720	NP_199086.2
Arabidopsis thaliana	At4g34480	NP_195174.6
Arabidopsis thaliana	At2g16230	NP_179219.4
Arabidopsis thaliana	At3g13560	NP_974303.1
Arabidopsis thaliana	At1g11820	NP_001184967.1
Arabidopsis thaliana	At1g66250	NP_176799.2
Arabidopsis thaliana	At2g01630	NP_001077866.1
Arabidopsis thaliana	At4g29360	NP_567828.3
Arabidopsis thaliana	At5g56590	NP_200470.1
Arabidopsis thaliana	At3g55780	NP_191137.1
Arabidopsis thaliana	At3g61810	NP_191740.1
Arabidopsis thaliana	At3g07320	NP_683538.1
Arabidopsis thaliana	At3g23770	NP_189019.1
Arabidopsis thaliana	At4g14080	NP_193144.1
Arabidopsis thaliana	At5g58480	NP_200656.2
Arabidopsis thaliana	At4g17180	NP_193451.2
Arabidopsis thaliana	At5g64790	NP_201284.1
Arabidopsis thaliana	At3g04010	NP_187051.3
Arabidopsis thaliana	At5g18220	NP_197323.1
Arabidopsis thaliana	At1g64760	NP_001031232.1

(Continued)

Table 1 | Continued

Organism	Identifier in this paper	Sequence identifier
Arabidopsis thaliana	At2g19440	NP_179534.1
Arabidopsis thaliana	At3g24330	NP_189076.1
Arabidopsis thaliana	At5g20870	NP_197587.1
Arabidopsis thaliana	At5g58090	NP_200617.2
Arabidopsis thaliana	At4g31140	NP_194843.1
Arabidopsis thaliana	At1g77790	NP_177902.1
Arabidopsis thaliana	At1g77780	NP_177901.1
Arabidopsis thaliana	At5g20390	NP_197539.1
Arabidopsis thaliana	At5g20560	NP_197556.1
Arabidopsis thaliana	At1g33220	NP_174592.1
Arabidopsis thaliana	At5g20340	NP_197534.1
Arabidopsis thaliana	At5g20330	NP_197533.1
Arabidopsis thaliana	At4g16260	NP_193361.4
Arabidopsis thaliana	At3g57270	NP_191286.1
Arabidopsis thaliana	At3g57240	NP_191283.2
Arabidopsis thaliana	At3g57260	NP_191285.1
Populus trichocarpa	PtGHL17_1	XP_002297638.2
Populus trichocarpa	PtGHL17_2	XP_002304004.2
Populus trichocarpa	PtGHL17_3	XP_002314794.2
Populus trichocarpa	PtGHL17_4	XP_002305879.1
Populus trichocarpa	PtGHL17_5	XP_006389594.1
Populus trichocarpa	PtGHL17_6	XP_006371969.1
Populus trichocarpa	PtGHL17_7	XP_002316783.2
Populus trichocarpa	PtGHL17_8	XP_002333242.1
Populus trichocarpa	PtGHL17_9	XP_002302861.2
Populus trichocarpa	PtGHL17_10	XP_002318439.2
Populus trichocarpa	PtGHL17_11	XP_006384505.1
Populus trichocarpa	PtGHL17_12	XP_006379239.1
Populus trichocarpa	PtGHL17_13	XP_002312097.1
Populus trichocarpa	PtGHL17_14	XP_002312098.1
Populus trichocarpa	PtGHL17_15	XP_002303070.2
Populus trichocarpa	PtGHL17_16	XP_002298356.1
Populus trichocarpa	PtGHL17_17	XP_002332000.1
Populus trichocarpa	PtGHL17_18	XP_002317055.2
Populus trichocarpa	PtGHL17_19	XP_002306003.2
Populus trichocarpa	PtGHL17_20	XP_006385314.1
Populus trichocarpa	PtGHL17_21	XP_002300505.2
Populus trichocarpa	PtGHL17_22	XP_002300634.2
Populus trichocarpa	PtGHL17_23	XP_002299750.2
Populus trichocarpa	PtGHL17_24	XP_002312820.1
Populus trichocarpa	PtGHL17_25	XP_002325214.2
Populus trichocarpa	PtGHL17_26	XP_002328249.1
Populus trichocarpa	PtGHL17_27	XP_002321273.1
Populus trichocarpa	PtGHL17_28	XP_006386924
Populus trichocarpa	PtGHL17_29	XP_002329975.1
Populus trichocarpa	PtGHL17_30	XP_002321266.1
Populus trichocarpa	PtGHL17_31	XP_002329954.1
Populus trichocarpa	PtGHL17_32	XP_002315222.2
Populus trichocarpa	PtGHL17_33	XP_002332466.1
Populus trichocarpa	PtGHL17_34	XP_002329964.1
Populus trichocarpa	PtGHL17_35	XP_002332467.1
Populus trichocarpa	PtGHL17_36	XP_002324127.1

(Continued)

Table 1 | Continued

Organism	Identifier in this paper	Sequence identifier
Populus trichocarpa	PtGHL17_37	XP_002329956.1
Populus trichocarpa	PtGHL17_38	XP_002302261.1
Populus trichocarpa	PtGHL17_39	XP_002313970.1
Populus trichocarpa	PtGHL17_40	XP_002319699.1
Populus trichocarpa	PtGHL17_41	XP_006372260.1
Populus trichocarpa	PtGHL17_42	XP_002330836.1
Populus trichocarpa	PtGHL17_43	XP_002308921.2
Populus trichocarpa	PtGHL17_44	XP_002306606.2
Populus trichocarpa	PtGHL17_45	XP_002299791.2
Populus trichocarpa	PtGHL17_46	XP_002309443.2
Populus trichocarpa	PtGHL17_47	XP_002310612.1
Populus trichocarpa	PtGHL17_48	XP_002323325.2
Populus trichocarpa	PtGHL17_49	XP_002314934.2
Populus trichocarpa	PtGHL17_50	XP_002315775.2
Populus trichocarpa	PtGHL17_51	XP_002308018.2
Populus trichocarpa	PtGHL17_52	XP_002314086.1
Populus trichocarpa	PtGHL17_53	XP_002324967
Populus trichocarpa	PtGHL17_54	XP_002305174.1

The table includes the source organism, abbreviation used in this study and sequence identifier en NCBI.

**This ORF was obtained by translating the sequence resulting from overlapping these two ESTs.*

(alignments provided in Supplementary data 2). In this case convergence was reached after 45000 generations.

A graphical representation of the GH17 domain alignment was performed using weblogo3 (Crooks et al., 2004). In the logo the overall height of the stack indicates the sequence conservation at that position.

GENERATION OF TRANSGENIC PLANT MATERIAL

Construction of p*35S*-mCitrine-PdBG1 (At3g13560) was described elsewhere (Benitez-Alfonso et al., 2013). N-terminal and GPI-anchor domains were predicted for At4g31140 and At5g58090 using SignalP 4.1 Serve and GPI-SOM (Fankhauser and Maser, 2005; Petersen et al., 2011). mCitrine protein fusions were obtained by overlapping PCR (Tian et al., 2004) and expressed in the binary vector pB7WG2.0 using Gateway technology. The mCitrine was fused in frame between amino acids 454–455 in the case of At4g31140 and between amino acids 445–446 in the case of At5g58090.

Transient expression was verified by agroinfiltration in *Nicotiana benthamiana* leaves. Stable transgenic lines were generated using the floral dip method, followed by selection with BASTA. T2 seeds were sterilized and germinated in long day conditions on plates containing MS medium supplemented with BASTA (25 μg/ml).

CALLOSE STAINING

Callose deposition at PD was detected in plant samples vacuum infiltrated with 0,1% (w/v) aniline blue in 0,1M sodium phosphate (pH 9.0) and incubated in the dark for 1–2 h before imaging.

Table 2 | Classification of embryophyte sequences based on protein structure and phylogenetic distribution.

Sequence identifier	Type	Branch
PpGHL17_1	1	α
PpGHL17_2	1	α
PpGHL17_3	1	α
PpGHL17_4	0	α
PpGHL17_5	1	α
PpGHL17_6	1	α
PpGHL17_7	2	α
PpGHL17_8	0	α
PpGHL17_9	0	α
PpGHL17_10	2	β
PpGHL17_11	1	α
PpGHL17_12	2	β
PpGHL17_13	2	β
PpGHL17_14	1	β
PpGHL17_15	1	β
PpGHL17_16	0	β
PpGHL17_17	0	α
PpGHL17_18	0	α
OsGHL17_1	3	α
OsGHL17_2	3	α
OsGHL17_3	3	α
OsGHL17_4	3	α
OsGHL17_5	3	α
OsGHL17_6	1	α
OsGHL17_7	3	α
OsGHL17_8	2	α
OsGHL17_9	2	α
OsGHL17_10	2	α
OsGHL17_11	2	β
OsGHL17_12	2	β
OsGHL17_13	2	β
OsGHL17_14	2	β
OsGHL17_15	2	β
OsGHL17_16	2	β
OsGHL17_17	2	β
OsGHL17_18	2	β
OsGHL17_19	2	β
OsGHL17_20	2	β
OsGHL17_21	2	β
OsGHL17_22	1	α
OsGHL17_23	3	α
OsGHL17_24	2	β
At2g05790	1	α
At4g26830	1	α
At5g55180	1	α
At4g18340	1	α
At1g30080	1	α
At2g26600	3	α
At3g15800	3	α
At2g27500	1	α
At5g42100	3	α

(Continued)

Table 2 | Continued

Sequence identifier	Type	Branch
At1g32860	3	α
At5g24318	1	α
At3g46570	1	α
At2g39640	1	α
At3g55430	1	α
At5g42720	3	α
At4g34480	1	α
At2g16230	1	α
At3g13560	2	α
At1g11820	1	α
At1g66250	2	α
At2g01630	2	α
At4g29360	2	α
At5g56590	2	α
At3g55780	1	α
At3g61810	1	α
At3g07320	1	α
At3g23770	1	α
At4g14080	1	α
At5g58480	2	β
At4g17180	1	β
At5g64790	2	β
At3g04010	2	β
At5g18220	2	β
At1g64760	2	β
At2g19440	2	β
At3g24330	2	β
At5g20870	2	β
At5g58090	2	β
At4g31140	2	β
At1g77790	1	γ
At1g77780	3	γ
At5g20390	1	γ
At5g20560	1	γ
At1g33220	1	γ
At5g20340	1	γ
At5g20330	1	γ
At4g16260	1	γ
At3g57270	1	γ
At3g57240	1	γ
At3g57260	1	γ
PtGHL17_1	2	α
PtGHL17_2	1	α
PtGHL17_3	2	α
PtGHL17_4	0	α
PtGHL17_5	2	α
PtGHL17_6	2	α
PtGHL17_7	1	α
PtGHL17_8	1	α
PtGHL17_9	1	α
PtGHL17_10	1	α
PtGHL17_11	1	α
PtGHL17_12	1	α
PtGHL17_13	1	α
PtGHL17_14	1	α
PtGHL17_15	1	α
PtGHL17_16	1	α
PtGHL17_17	1	α
PtGHL17_18	3	α
PtGHL17_19	1	α
PtGHL17_20	3	α
PtGHL17_21	3	α
PtGHL17_22	1	α
PtGHL17_23	3	α
PtGHL17_24	1	α
PtGHL17_25	3	α
PtGHL17_26	3	α
PtGHL17_27	1	α
PtGHL17_28	1	α
PtGHL17_29	3	α
PtGHL17_30	1	α
PtGHL17_31	1	α
PtGHL17_32	2	α
PtGHL17_33	1	α
PtGHL17_34	2	α
PtGHL17_35	1	β
PtGHL17_36	2	β
PtGHL17_37	1	α
PtGHL17_38	0	γ
PtGHL17_39	2	β
PtGHL17_40	2	β
PtGHL17_41	2	β
PtGHL17_42	1	β
PtGHL17_43	1	γ
PtGHL17_44	0	γ
PtGHL17_45	1	γ
PtGHL17_46	2	β
PtGHL17_47	2	β
PtGHL17_48	1	γ
PtGHL17_49	1	γ
PtGHL17_50	0	γ
PtGHL17_51	1	γ
PtGHL17_52	1	γ
PtGHL17_53	2	β
PtGHL17_54	3	α

The table classifies the sequences used in this paper according to the presence of signal peptide, X8 domain and/or GPI anchor as described in Materials and Methods. It also mentions the branch in the tree where this sequence appears. Consult ***Table 1*** *to access the sequence corresponding to each identifier in NCBI.*

MICROSCOPY

Confocal analysis was performed on a Zeiss LSM700 Inverted microscope using a 488 nm excitation laser for mCitrine, the 405 nm laser for aniline blue fluorochrome and 585 nm laser to detect chloroplast autofluorescence. Emission was collected using the filters: BP 505–530 for mCitrine, the DAPI filter for aniline

blue (463 nm) and LP 615 filter for chloroplasts (581 nm). The images corresponded to stacks of z- optical sections. Sequential scanning was used to image tissues expressing mCitrine and stained with aniline blue.

RESULTS

IDENTIFICATION OF GHL17 SEQUENCES IN CHAROPHYTES AND EMBRYOPHYTES SUGGEST GENE FAMILY EXPANSION

The presence of intercellular connections (phragmoplast and/or less evolved PD) has been described in some species belonging to the *Charophytes* (**Figure 1**) but so far, in this lineage, regulation of PD by callose metabolism has only been demonstrated in embryophytes (Scherp et al., 2001; Schuette et al., 2009). The presence of β-1,3 glucans in the cell wall of unicellular organisms indicate an ancient origin for this metabolic pathway but how and when it evolved to control PD transport is unknown (Sorensen et al., 2011). In an attempt to answer this question, we isolated sequences encoding GH17 domains from charophytes, bryophytes, and vascular plants. Based on the availability of sequence information, we selected representative species from the charophycean orders: Klebsormidiales (*Klebsormidium flaccidum*), Zignematales (*Penium margaritaceum*), Coleochatales (*Chaetosphaeridium globosum*) and Charales (*Nitella mirabilis*). 14 partial transcripts were isolated but only 7 (2 from *Klebsormidium,* 1 from *Penium*, 1 from *C. globosum* and 3 from *Nitella*) contained key aminoacids forming the active site of GHL17 (**Table 1**).

Full-length GHL17 sequences were isolated from moss (*Physcomitrella patents*) and from monocots (*Oryza sativa*) and dicots (*Arabidopsis thaliana* and *Populus trichocarpa*) model plants using genome information and protein annotation databases. In total we were able to identify 18 sequences in *Physcomitrella*, 24 sequences in *Oryza sativa,* 50 sequences in *Arabidopsis thaliana* and 54 in *Populus trichocarpa* (**Table 1**). The increasing number of sequences isolated in land plants with respect to those isolated in algae and moss suggests that an expansion in this gene family have occurred during or immediately after land colonization.

We used prediction tools to determine the structure and localization of the proteins encoded by the sequences identified. This was not possible for algae representatives because only partial transcripts were isolated. For moss, rice, Arabidopsis and Populus sequences, secretory signal peptides (SP) and the presence of C-terminal GPI anchoring domains were predicted using several bioinformatics websites (see Material and Methods). GHL17 sequences were also classified according to the presence of one or more carbohydrate binding domains (named X8 or CBM43). We classified sequences in 4 types according to the presence of one or more of these features (see Material and Methods and **Table 2**). Type 2 and 3 displayed a SP and GPI-anchor signature that predicts their localization at the PM or at membranous subdomains (such as PD). From the 18 sequences isolated in *Physcomitrella* only 4 were classified as type 2. Arabidopsis genome contained 21 membrane predicted sequences (42% of the total), which were experimentally verified in a proteomic analysis (Borner et al., 2003). The number of membrane predicted GHL17 was very similar in rice and *Populus trichocarpa* (22 in rice, 21 in poplar). When comparing moss and vascular plants a major increase in the number of predicted membrane-targeted proteins is detected consistent with the hypothesis that GHL17 evolved and expanded to support or adopt specialized functions at membraneous domains in terrestrial environments.

KEY AMINO ACID RESIDUES IN THE GH17 DOMAIN ARE CONSERVED THROUGHOUT EVOLUTION

Research on GHL17 protein structure revealed two strictly conserved glutamate residues that act as the proton donor and the nucleophile in all reactions catalyzed by glycosyl hydrolases (Jenkins et al., 1995; Wojtkowiak et al., 2013). A number of aromatic and hydrophilic residues located near the catalytic cleft, presumably involved in substrate specificity and enzyme activity, are also conserved among all plant GHL17 proteins (Wojtkowiak et al., 2013).

To study the molecular evolution of the GH17 domain in green algae, moss and plants, we translated and aligned the domain region of the retrieved sequences using MEGA5 (Supplementary data 1). We also included sequences isolated from fungi representatives to analyze domain conservation in a different lineage. The results revealed that the glutamate catalytic residues (E) are highly conserved among all charophycean representatives, fungi and embryophytes (highlighted in red in the alignment shown in Supplementary data 1 and in **Figure 2**). Similarly, the residues surrounding the catalytic site are mostly conserved in all selected representatives (Supplementary data 1, **Figure 2**). Moreover a region contained the aromatic residues Tyr200 and Phe203 (location refer to At2g05790 sequence), which is involved in substrate interaction (Wojtkowiak et al., 2013), is also conserved in all streptophytes (**Figure 2**).

The high degree of similarity between the catalytic sites of GHL17 proteins in green algae, fungi and land plants supports the ancestral origins of this metabolic pathway.

PHYLOGENY REVEALED A GROUP OF GHL17 PROTEINS THAT APPEARED IN EMBRYOPHYTES ONLY

The phylogenetic distribution of Arabidopsis GHL17 sequences has been studied before (Doxey et al., 2007). Based on tree topology, these proteins were grouped into three distinct clades: α, β, and γ. Predicted membrane GHL17 were evenly distributed in clade α and β. We investigated the evolutionary origin of these clades by comparing the phylogenetic distribution of GHL17 sequences isolated from charophycean green algae, fungi *Physcomitrella patens*, *Oryza sativa* and *Arabidopsis thaliana*. Although plants and fungi evolved in a different lineage, they share a common eukaryotic origin, which is reflected in the conservation of key aminoacids in the GH17 domain (Supplementary data 1).

Unrooted phylogenetic trees were generated using three search algorithms: Bayesian inference (Bayesian), Maximum Likelihood (ML) and Neighbor Joining (NJ) (**Figure 3A** and supplementary data 3). The tree topology was generally well supported by all 3 methods, with the exception of several higher order branches in ML and NJ bootstrap values. The three phylogenetic clades (α, β, and γ) described by Doxey et al. (2007) are color coded in **Figure 3A**. Fungi selected sequences branch off at the same

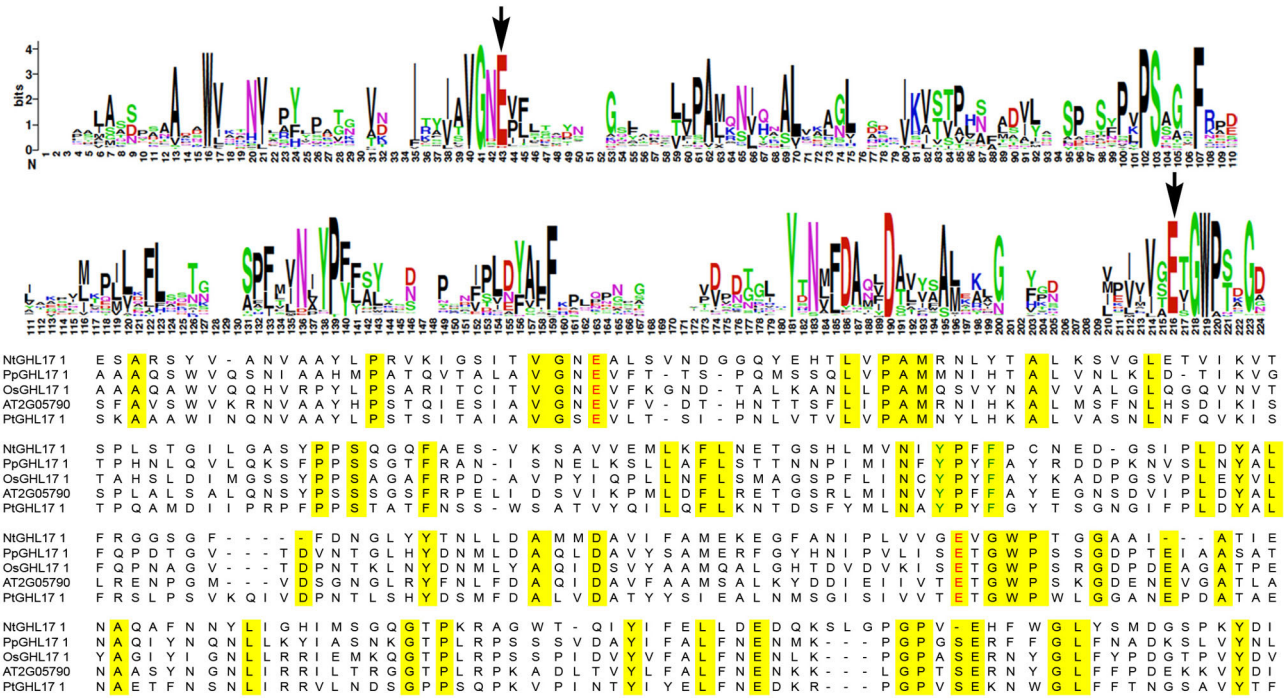

FIGURE 2 | Sequence conservation in the domain region of GHL17 proteins. The top panels show the consensus region for GH17 using weblogo. This was obtained by aligning all the sequences isolated from green algae and embryophytes (consult **Table 1** to obtain the NCBI identifier for these sequences). The bottom panel shows an alignment of representative domain sequences from *Nitella mirabilis* (NtGHL17_1), from moss (PpGHL17_1) and from the vascular plants *Arabidopsis thaliana* (At2G05790), *Oryza sativa* (OsGHL17_1) and *Populus trichocarpa* (PtGHL17_1). Conserved aminoacids are highlighted in yellow in the alignment. The position of the glutamate residues (E) actively involved in the catalytic reaction is indicated with arrows in the weblogo and in red in the alignment. Notice conserved domains around the catalytic sites. Tyr (Y) and Phe (F) residues conserved in plants and presumably important in substrate binding are indicated in green in the bottom panel.

point as some algae representatives and near the point of connection of plant sequences forming the clade beta. This suggests a more ancestral origin for this clade (**Figure 3B**). Clade alpha and gamma contained embryophytes only and, for the purpose of this paper, they could be considered as a single clade (**Figure 3C**).

Only partial transcripts were isolated for algae representatives hence gaps were introduced in the alignments that could affect the accuracy and reliability of the trees. To confirm the tree topology, we manually eliminate these gaps to generate trees containing the sequence region encoding the domain only (marked in yellow in Supplementary data 1). As shown in supplementary data 3, the distribution of sequences in the different clades and the relationship between the different branches was conserved in these "domain only" trees.

As in Arabidopsis, even distribution of predicted membrane sequences between the alpha and the beta clade was observed in rice (**Figures 3B,C**). Interestingly, type 3 proteins were almost exclusively found in the alpha clade. In summary our phylogenetic analysis suggest that GHL17 membrane proteins contained in clade alpha appeared in early embryophytes presumably to adopt new functions at the cell periphery.

PD LOCALIZED GHL17 PROTEINS ARE CONTAINED IN THE α CLADE

Since cell wall composition and PD complexity evolved during land plant colonization, it seems logical to assume that callose, and specialized callose metabolic enzymes, were adopted at some stage during this evolutionary process to regulate PD aperture. The presence of charophytic sequences and the proximity to a fungi branch suggests a more ancestral origin for membrane proteins included in the beta clade (**Figure 3B**). We hypothesize that PD-targeted GHL17 proteins evolved with the appearance of early embryophytes, hence likely be contained within the alpha clade (**Figure 3C**).

The Bayesian tree shows (with high support values) 10 predicted membrane proteins (type 2 and 3) from Arabidopsis contained in the alpha clade whereas 10 type 2 sequences appeared in a compact clade within the beta subgroup surrounded by sequences isolated from green algae (**Figures 3B,C**). Data from several publications reported the intracellular localization of several GHL17 proteins in Arabidopsis. The root developmental regulators At3g13560, At2g01630, and At1g66250 (Benitez-Alfonso et al., 2013) and the virus-induced protein At5g42100 (Levy et al., 2007) were PD-localized whereas At3g57260 was preferentially expressed in the apoplast (Zavaliev et al., 2013). Confirming our hypothesis, all PD localized proteins were grouped in the alpha clade (**Figure 3C**).

The localization of few GHL17 proteins from Populus has been recently reported (Pechanova et al., 2010; Rinne et al., 2011). To test the relationship between the appearance of the alpha clade and protein localization, we constructed a Bayesian tree with

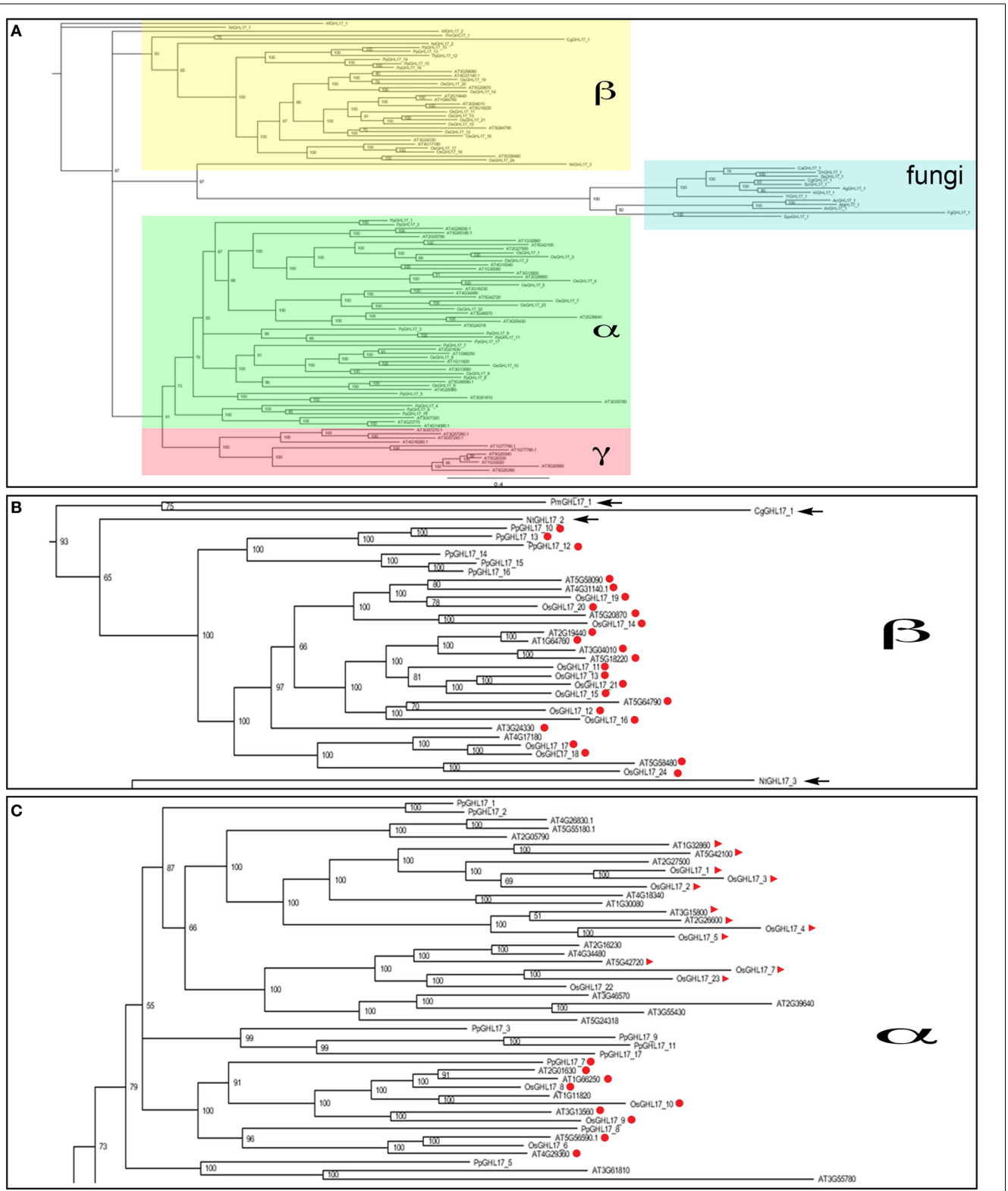

FIGURE 3 | Bayesian phylogenetic consensus tree of GHL17 sequences isolated from fungi, green algae and embryophytes representatives (A). All sequences are cited in **Table 1** and alignment provided in Supplementary data 1. Bayesian posterior probabilities are indicated in the branches. Clades α (in green), β (in yellow), and γ (in red), as defined for Arabidopsis in Doxey et al. (2007), are indicated. Fungi sequences form a separate group consistent with a different evolutionary lineage. **(B)** shows a close-up of clade β and **(C)** shows a portion of the α clade. Algae sequences are arrowed in **(B)** and membrane predicted proteins, type 2 and 3, are marked in red circles and red triangles respectively.

GHL17 sequences isolated from Arabidopsis and from *Populus trichocarpa*. BLAST searches against the Populus genome identified a total of 54 non-redundant sequences containing the GH17 domain (**Table 1**). Classification of these sequences according to bioinformatic predictions identified 21 putative membrane proteins (**Table 2**). A multiple sequence alignment was conducted and unrooted phylogenetic trees were generated using the Bayesian, ML and NJ algorithms (**Figure 4** and Supplementary data 2 and 4). According to tree topology, Populus GHL17 proteins also appeared grouped in 3 clades α, β, and γ, each well supported by high probability values in each tree (**Figure 4** and Supplementary data 4). As before, type 3 proteins were contained within the α clade whereas type 2 proteins were distributed between the α and β clades.

Orthologs of PtGHL17_18 and PtGHL17_26 were both found to target PD whereas PtGHL17_48 and PtGHL17_49 orthologs were mainly localized at the PM and lipid bodies (Rinne et al., 2011). As expected, PtGHL17_18 and PtGHL17_26 are membrane predicted proteins contained in the alpha clade (**Figure 4**). The results confirmed a potential link between the phylogenetic distribution of GHL17 proteins and their intracellular localization.

USING PHYLOGENETIC DISTRIBUTION TO DISCRIMINATE BETWEEN CANDIDATES FOR PD LOCALIZATION

To identify novel PD components the proteomic composition of PD-enriched cell walls has been analyzed (Bayer et al., 2006; Fernandez-Calvino et al., 2011). Several GHL17 proteins were isolated through these screens, including the predicted membrane localized proteins At3g13560, At5g42100, At4g31140, and At5g58090. Different from At3g13560 and At5g42100 (included in the alpha clade), At4g31140 and At5g58090 were found in clade beta. Successful separation of PD membranous section from the desmotubule and the PM is quite challenging (if not impossible) therefore a number of false positives was expected. The results presented above suggest that proteins excluded from the alpha clade are not likely targeted to PD sites. Therefore, we tested the intracellular localization of At4g31140 and At5g58090 using as control At3g13560-mCitrine (a previously PD-localized GHL17 protein). m-Citrine fluorescent fusions were obtained and expressed transiently in tobacco leaves. The results are shown in **Figure 5**. Transient expression of either At4g31140-mCit or At5g58090-mCit led to protein accumulation in the apoplast (**Figures 5A–C**). At5g58090-mCit also appears to be associated with the endoplasmic reticulum (data not shown).

Transient assays can be misleading. Therefore we obtained stable transgenic lines expressing p*35s*-At5g58090-mCit to confirm the subcellular localization of this protein. Leaves isolated from 10 days-old seedlings expressing p*35s*-At5g58090-mCit and leaves isolated from seedlings overexpressing At3g13560-mCit (grown in the same plate) were stained with aniline blue to reveal callose deposits at PD sites. The intracellular localization of these proteins in stable lines reproduced the results obtained in transient assays (**Figures 5D,E**): At5g58090-mCit was found at the cell periphery and in the apoplast whereas At3g13560-mCit was found in a punctuated pattern along the cell wall (presumably PD sites). Co-localization with callose deposits at PD was found for At3g13560 but not for At5g58090 (white arrows in **Figures 5D,E**). This result suggests that PD localization of GHL17 proteins could be related to their evolutionary origin, hence with the appearance of the alpha clade.

DISCUSSION

GHL17 proteins play many different roles in plant development and response to biotic and abiotic stresses (Doxey et al., 2007). Functional specialization can be predicted by studying protein sequence, gene expression and phylogeny (Doxey et al., 2007). Here, we used phylogenetic tree reconstruction to study when in land plant evolution GHL17 membrane proteins diversify to play a role at PD. First, we identified sequences encoding for a GH17 domain in representatives of green algae, fungi, bryophytes and vascular plants. Fungi, as plants, deposit callose at the cell wall but don't form plasmodesmata connections. Therefore they are an ideal organism to analyze the evolution of 1,3 beta glucanases in a different lineage.

Study of the protein sequences isolated suggests that the key amino acids involved in GH17 catalytic activity are highly conserved throughout evolution. This is in agreement with other reports that demonstrate the presence of beta 1,3 glucans in the cell wall of ancient unicellular algae where it is required for cell division and cell wall biogenesis (Scherp et al., 2001; Sorensen et al., 2011). Specialization of GHL17 proteins to play specific roles in the control of PD transport is therefore likely a consequence of evolutionary functional diversification within this family.

Classification of embryophytes GHL17 proteins according to the presence or absence of a signal peptide, of a GPI-anchored domain and of one or more carbohydrate binding domain (X8) predicted PM or PD localization for a set of proteins. The number of membrane predicted proteins increased from 4 identified in moss to 21–22 identified in vascular plants suggesting that an expansion occur in this protein family during land plant evolution. This might have been necessary to support the adaptation of multicellular organism to terrestrial environments, which might require specialized GHL17 proteins to assume divergent or redundant functions at the PM or membraneous subdomains.

Using phylogenetic analysis we found that membrane-targeted sequences are evenly distributed in two major clades (**Figure 3**). Clade alpha contained GHL17 sequences that appeared in embryophytes only whereas the beta clade comprised land plants and algae proteins and is closely related to a branch containing fungi sequences. This result suggest that clade alpha evolved early during land colonization in the Streptophyte lineage, whereas clade beta is form by proteins of a more ancestral origin (**Figures 3B,C**). Ultrastructural studies revealed the accumulation of callose at PD sites in early embryophytes (Scherp et al., 2001; Schuette et al., 2009) therefore GHL17 proteins participating in the regulation of callose at PD sites will likely appear in clade alpha.

Indeed, we noticed that all Arabidopsis PD-located GHL17 proteins (identified up to date) are clustered in the alpha clade. This established an interesting link between the phylogenetic distribution of GHL17 proteins and their intracellular localization. This correlation was confirmed in Populus: membrane proteins

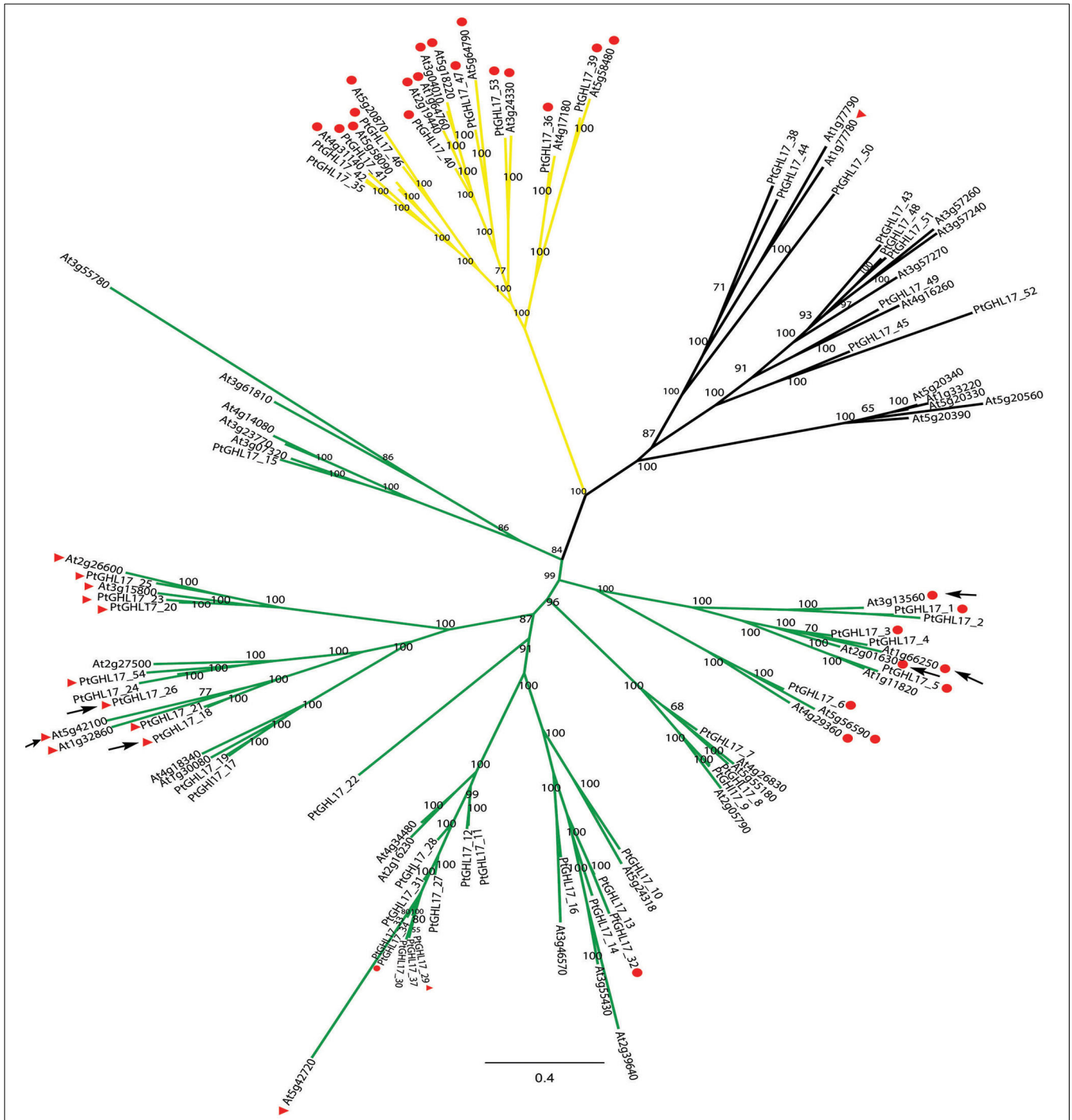

FIGURE 4 | Majority consensus tree generated by Bayesian inference of phylogeny of GHL17 proteins isolated from *A. thaliana* (At) and *P. trichocarpa* (Pt) (sequences cited in Table 1). Bayesian posterior probabilities are indicated in the tree branches. In accordance with the phylogenetic tree presented in **Figure 3**, branches forming clades α (green), β (yellow) and γ (black) have been indicated. Type 2 and 3 proteins (GPI-anchored proteins) are indicated with red circles and red triangles respectively. The position of PtGHL17_18 and PtGHL17_26 (reported to localize at PD by Rinne et al., 2011), as well as the position of PD-localized Arabidopsis proteins has been indicated with arrows.

belonging to the alpha clade were reported to localize at PD but this was not the case for proteins contained in other clades (Rinne et al., 2011). We tested the use of this knowledge for the discrimination of false positives isolated in a proteomic screen of Arabidopsis PD. Two proteins from the beta clade were identified in the PD proteome but intracellular localization of mCitrine protein fusions revealed that they accumulate in the apoplast (**Figure 5**). Our results suggest that phylogenetic analysis could

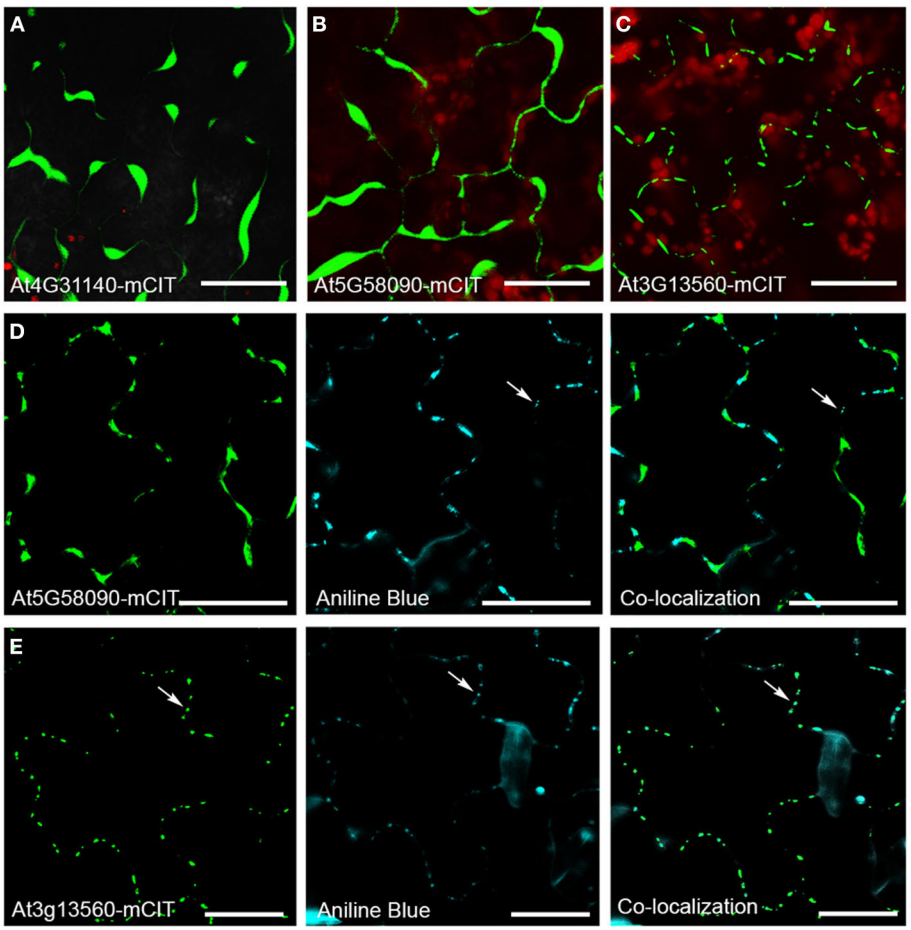

FIGURE 5 | Intracellular localization of GHL17 protein m-Citrine fusions. (A,B,C) Show At4g31140-mCit, At5g58090-mCitm, and At3g13560-mCit transient expression in tobacco leaves. Chloroplast auto-fluorescence appears in red. **(D,E)** Show At5g58090-mCit and At3g13560-mCit fluorescence (green) in Arabidopsis leaves expressing the fusion proteins under the 35S promoter. Aniline blue staining of callose deposits (blue) and the green and blue channels superimposed are also shown. Notice that At3g13560 expression, but not At5g58090, co-localizes with callose deposits at PD (white arrows). Scale bars = 20 μm.

be potentially a useful tool for the preliminary detection of false positive when screening for PD-localized GHL17 proteins.

To summarize, the results obtained so far suggest that, during (or immediately after) colonization of terrestrial habitats by streptophytes, GHL17 gene family evolved and expanded to play specialized roles at the cell membrane, including PD regulation. Completion of genome sequence and further studies on callose regulation in ancestral charophyceans will be essential to confirm or refute this theory. Study of phylogenetic relationships between ancestral PM targeted GHL17 and those that evolved with embryophytes was used here to discriminate between PD-localized and non PD-localized proteins in Arabidopsis and Populus. This knowledge could theoretically be applied to the preliminary screening of GHL17 proteins (aiming to identified those that serve specialized roles are PD sites) in other land plant representatives.

AUTHOR CONTRIBUTIONS

Rocio Gaudioso-Pedraza performed the research, analyzed the data and designed the Figures. Yoselin Benitez-Alfonso designed the experiments, wrote the manuscript, performed research and interpreted the data for the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00212/abstract

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Plasmodesmata-mediated intercellular signaling during plant growth and development

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Plasmodesmata (PD) are cytoplasmic channels that connect neighboring cells for cell-to-cell communication. PD structure and function vary temporally and spatially to allow formation of symplastic domains during different stages of plant development. Reversible deposition of callose at PD plays an important role in controlling molecular trafficking through PD by regulating their size exclusion limit. Previously, we reported several semi-dominant mutants for *CALLOSE SYNTHASE 3* (*CALS3*) gene, which overproduce callose at PD in *Arabidopsis*. By combining two of these mutations in a LexA-VP16-ER (XVE)-based estradiol inducible vector system, a tool known as the "*icals3m* system" was developed to temporally obstruct the symplastic connections in a specified spatial domain. The system has been successfully tested and used, in combination with other methods, to investigate the route for mobile signals such as the SHR protein, microRNA165/6, and cytokinins in *Arabidopsis* roots, and also to understand the role of symplastic domain formation during lateral root development. We envision that this tool may also be useful for identifying tissue-specific symplastic regulatory networks and to analyze symplastic movement of metabolites.

Keywords: plasmodesmata (PD), non-cell autonomous proteins (NCAP), plant development, callose, symplastic domains, size exclusion limit (SEL)

INTRODUCTION

In plants, the exchange of information between cells is essential for their growth, response to the environment and defense. During development, the transmission of positional signals between different cells, tissues and organs is required for the determination of their identities. These signals include hormones, metabolites, non-cell autonomous proteins and RNAs, which can move either through the process of exocytosis and endocytosis (apoplastic signaling) or via Plasmodesmata (PD) (symplastic signaling). PD connect the cytoplasms of plant cells and act as channels for trafficking of signaling molecules, which can pass either via simple diffusion (non-targeted movement) or by temporarily changing PD diameter (targeted movement). Here, we are discussing symplastic signaling and the role of callose during plant development and describing a tool which can be used to temporally obstruct molecular trafficking through PD in a tissue-specific manner to understand the role of symplatic communication in plant developmental processes.

PD STATES DEFINE SPATIAL SYMPLASTIC DOMAINS

Plasmodesmata are developed across the cell walls to enable cytoplasmic connection and molecular trafficking between neighboring cells. PD channels are lined by plasma membrane at their boundaries, and the desmotubule (DT), a structure composed of compressed endoplasmic reticulum (ER), is located in the center of the pores. The region between the plasma membrane and the DT is known as the cytoplasmic sleeve (CS), which provides a major path for molecular movement through PD. A large number of different kinds of proteins associated with PD have been identified using proteomic and biochemical approaches (Bayer et al., 2006; Levy et al., 2007; Thomas et al., 2008; Simpson et al., 2009; Fernandez-Calvino et al., 2011; Ham et al., 2012; Salmon and Bayer, 2013). Some of these proteins such as PDLP1 are uniformly distributed along the plasma membrane (Thomas et al., 2008), whereas others may be specifically localized to the regional membrane microdomains (Simpson et al., 2009). Therefore, presence of membrane microdomain-associated proteins at PD raises a possibility that a special membrane microdomain is associated with PD (Raffaele et al., 2009; Mongrand et al., 2010) that may act as a sorting platform for recruitment of PD-associated proteins (Mongrand et al., 2010; Simon-Plas et al., 2011; Tilsner et al., 2011).

PD can exist in different states depending on their permeability during plant growth and development. Closed PD do not permit any trafficking, whereas small molecules such as ions, photoassimilates and growth regulators can diffuse through opened PD. Apart from closed/open state, PD can also be in a dilated state in different tissues to allow movement of larger molecules. A dilation state of the PD is defined by their size exclusion limit (SEL) which is the upper size limit of the molecules that can move through PD. The SEL of PD varies in different cells and tissues. For example, PD located on stele/endodermis and cortex/epidermis boundaries have SEL ~60 kDa whereas PD connecting companion cells (CC) and sieve elements (SE) generally have SEL >67 kDa (Stadler et al., 2005; Rim et al., 2011). During various stages of differentiation, a dynamic control over PD permeability allows formation of some segregated regions, called "symplastic domains" in which communication among the cells is free, while between the domains, it is restricted (Rinne and Van Der Schoot, 1998; Gisel et al., 1999). These functional domains, therefore, allow specific developmental programs to take place in restricted areas. For

example, the early staged embryo constitutes a single symplast due to opened interconnection between the cells, but at the later stages of development, PD change their SEL to generate distinct symplastic domains as shown by the movements of different sized tracers (Kim et al., 2005a,b).

SYMPLASTIC SIGNALING DURING SHOOT AND ROOT DEVELOPMENT

A large number of critical cell identity regulators, non-cell autonomous transcription factors and small RNAs have been reported to traffic between cells. The first discovered mobile regulator was *KNOTTED1 (KN1)*, which regulates formation and maintenance of the shoot apical meristem (SAM) in maize (Jackson et al., 1994; Lucas et al., 1995). Subsequently, the movement of *Arabidopsis* homologs of *KN1*, *KNOTTED*1-like homeobox protein 1/*BREVIPEDICELLUS (KNAT1/BP)* and *SHOOTMERISTEMLESS (STM)* from L1 to L2/L3 layers of the SAM was shown in *Arabidopsis* (Kim et al., 2003). Yet another homeodomain transcription factor, *WUSCHEL (WUS)* moves from the organizing center to the adjacent cells of the SAM and activates *CLAVATA 3* (*CLV3*), which inturn represses *WUS* expression with *CLV1*, forming a feedback loop to control the size of the SAM (Schoof et al., 2000; Yadav et al., 2011).

The long distance movement of *FLOWERING LOCUS T (FT)* from the leaves to the shoot apex via phloem to promote *LEAFY* (*LFY*) expression is required to induce flowering (Corbesier et al., 2007; Mathieu et al., 2007). *LFY* also functions non-cell autonomously by moving to adjacent cells through PD to activate downstream target genes (Sessions et al., 2000, Wu et al., 2003). Additionally, some MADS-box transcription factors exhibit non-cell autonomous functions during floral organ patterning. *Antirrhinum* B-function factors, *DEFICIENS* (*DEF*) and *GLOBOSA (GLO)*, have been shown to exhibit regulated mobility (Perbal et al., 1996). In *Arabidopsis*, the C-function gene *AGAMOUS* (*AG*) can move from the epidermal cell layer to the subepidermal cell layer of the floral meristem through secondary PD (Urbanus et al., 2010).

In *Arabidopsis*, the quiescent center (QC) and columella cells of the root derive from the hypophysis, and other cells develop from the embryo proper (Dolan et al., 1993). The auxin response factor *MONOPTEROS (MP)* activates the expression of *TARGET OF MONOPTEROS 7* (*TMO7*) in embryonic cells, and the *TMO7* protein moves to the hypophysis precursor to promote its asymmetric division (Schlereth et al., 2010). For continuous growth and development of the root, several signaling events that balance cell division and cell differentiation are required. *SHORTROOT* (*SHR*) is expressed in the stele cells but the protein migrates to the neighboring cell layer (the QC, the cortex/endodermal initial and the endodermis). Activation of *SCARECROW (SCR)* expression by SHR in the QC is critical for specifying the QC cells and maintaining surrounding initials (Helariutta et al., 2000; Nakajima et al., 2001). In the cortex/endodermal initials (CEIs), SHR/SCR regulates the expression of a cell-cycle regulator, *CYCLIN D6*;*1* to trigger the asymmetric cell division (Sozzani et al., 2010). *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)*, is expressed in the QC and like its SAM homologue *WUS*, *WOX5* non-cell-autonomously maintains columella stem cells (CSC) in the root niche (Sarkar et al., 2007), suggesting that either WOX5 itself or its downstream components move from QC to columella initials. Additionally, *ARABIDOPSIS CRINKLY4 (ACR4)* and *CLV1* assemble into a complex to perceive the *CLAVATA3/EMBRYO SURROUNDING REGION40 (CLE40)* signal and restrict the expression of *WOX5* to control the distal root meristem (Stahl et al., 2009, 2013). Interestingly, both *ACR4* and *CLV1* can interact at PD, suggesting that they may have a role in regulating the trafficking through PD (Stahl et al., 2013).

The radial patterning of the root vascular tissues relies on a bi-directional signaling between the stele and the endodermis. SHR protein moves from the stele into the endodermis and together with SCR it activates the expression of microRNA165/6. The miR165/6 then moves in the opposite direction into the vascular tissues and establishes a concentration gradient for their targets, the *HD-ZIP III* genes (Carlsbecker et al., 2010). The miRNA-dependent post-transcriptional regulation of *PHB* expression is required for xylem specification and pericycle differentiation and also to maintain the expression of *JACKDAW (JKD)* in the ground-tissue (GT), the endodermis and the cortex, to restrict *SHR*, and *SCR* movement (Miyashima et al., 2011).

CALLOSE PLAYS AN IMPORTANT ROLE IN REGULATING SYMPLASTIC COMMUNICATION DURING PLANT GROWTH AND DEVELOPMENT

Symplastic communication in plants is largely regulated through a control on the SEL of PD either by developmental or environmental factors. Callose is one of these factors that play an important role in regulating inter-cellular communication through PD in a wide range of developmental and physiological processes (Chen and Kim, 2009). It is biosynthesized by callose synthases (*CALS*, also called glucan synthase-like, GSL; Verma and Hong, 2001) and dynamically deposited during cell plate formation in dividing cells, during pollen development and pollen tube growth and to some specialized cell-wall domains such as PD and sieve plates of phloem SE. Its degradation, on the other hand, is controlled by activities of callose degrading enzymes called β-1, 3-glucanases (BGs). Therefore, a balance between these metabolic enzymes regulates callose levels in plant cells (Chen and Kim, 2009; Zavaliev et al., 2011).

Despite of a large genetic redundancy among the *CALS/GSL* members, some of these genes have been shown to be involved in specific processes. For example, *CALS10* (*GSL8*) has a role during cytokinesis, stomata patterning and ploidy consistency in gametes (Chen et al., 2009; Guseman et al., 2010; De Storme et al., 2013), whereas SE-specific gene, *CALS7 (GSL7)* is required for callose deposition at PD and the sieve plates of sieve cells (Barratt et al., 2011; Xie et al., 2011). *CALS3 (GSL12)* has a broad expression domain in *Arabidopsis* root, and the protein is localized to the plasma membrane and PD (Vatén et al., 2011). *cals3-d* gain-of-function mutants have increased level of callose at PD, resulting in pleiotropic developmental defects (Vatén et al., 2011). Similarly, BGs are also involved in a wide range biological processes including development, stress responses and pathogen defense (Doxey et al., 2007). For example, in *Arabidopsis*, AtBG_ppap controls molecular trafficking through PD and *PdBG1/PdBG2* play an important role

during lateral root (LR) development (Levy et al., 2007; Benitez-Alfonso et al., 2013). However, in tobacco, a *CLASS I BETA-1,3-GLUCANASE* (β*GLU1)* is induced during seed germination and releases them from dormancy (Leubner-Metzger and Meins, 2000, 2001). In addition to the callose synthases and glucanases, several other genes also regulate symplastic trafficking by affecting callose levels (Thomas et al., 2008; Simpson et al., 2009; Lee et al., 2011). Collectively, these studies suggest that critical level of callose is required during plant development and various environmental conditions.

ROLE OF CALLOSE IN CELLULAR ISOLATION AND SYMPLASTIC DOMAIN FORMATION DURING DEVELOPMENT

While PD provide an important path for cell-to-cell communication, regulation of their SEL at the same time also ensures a certain level of cell individuality by restricting the diffusion of certain larger factors through PD (Oparka, 1993). Some cells become even fully symplastically isolated after differentiation either by losing their PD (e.g., guard cells) or by severely restricting molecular trafficking (e.g., root cap) through PD (Erwee and Goodwin, 1985; Palevitz and Hepler, 1985; Oparka, 1993). However, formation of symplastic domains often does not require a complete closure or loss of PD, since a temporal modulation of PD permeability can be enough for creation of these functional domains during development (Epel and Bandurski, 1990). Reversible deposition of callose provides an important mechanism of control over PD in symplasmic organization. For example, in poplar and birch SAMs, callose deposition results in a closure of PD during dormancy period, which eventually is restored by β-1, 3-glucanases during chilling-induced dormancy release (Rinne et al., 2001, 2011). During stomata patterning in Arabidopsis, callose creates a local sub-domain for stomata-specific developmental programs to take place by restricting the stomata identity factor, *SPEECHLESS (SPCH)* only to the stomata initials. In cals10 mutants, stomata are developed in clusters as a result of enhanced movement of *SPCH* to neighboring cells due to increased symplastic connectivity (Guseman et al., 2010). In Arabidopsis roots, callose level controls symplastic domains in the root meristem and LR primordia. Free GFP expressed under the phloem CC specific AtSUC2 promoter is symplastically released from the CC traffics predominantly through the SE, and diffuses freely into the root tip (Imlau et al., 1999). This diffusion of free GFP is decreased in callose accumulating gfp arrested trafficking 1 (*gat1*) and *cals3-d* mutants (Benitez-Alfonso et al., 2009; Vatén et al., 2011). *GAT1* encodes for an m-type thioredoxin that controls symplastic permeability by controlling redox regulation of callose deposition in the root meristem. In Arabidopsis LRs, the callose deposition at PD correlates with symplastic domain formation during LR primordia specification and influences the initiation and patterning of LRs (Benitez-Alfonso et al., 2013). Thus, callose-mediated regulation of SEL of PD is important for creation of symplastic domains during plant development.

THE *icals3m* SYSTEM; A TOOL TO CONTROL MOLECULAR TRAFFICKING THROUGH PD

Although a large number of non-cell autonomous signals that control plant development have been identified, only little is known about how these signals move between the cells. This is to an extent due to absence of any suitable tool to study molecular trafficking through PD. We have recently developed a system, *icals3m,* to obstruct trafficking through PD by over-producing callose in the vicinity of PD (Vatén et al., 2011). In this system, a tissue-specific estradiol-inducible LexA-VP16-ER (XVE) system regulates the expression of a mutant *cals3m* gene in a specified spatial domain by using either a specific promoter or an enhancer-trap line, thus enabling a temporal and spatial control on callose production (Zuo et al., 2000; Vatén et al., 2011). Interestingly, the over-produced callose is not only deposited to the neck region of PD, but also along the entire PD channels, allowing a uniform closure of the channel (Vatén et al., 2011).

APPLICATION OF THE *icals3m* IN STUDYING INTER-CELLULAR TRAFFICKING OF PROTEINS AND SMALL RNAs

The specificity and efficiency of the system has been demonstrated in various tissues by multiple studies focusing on different biological processes. When the *cals3m* is induced in the GT-specific enhancer line (*J0571; p6xUAS::icals3m*), a high level callose is produced in the endodermis and cortex (**Figures 1A,B**), causing a hindrance in the symplastic connectivity between the endodermis and the stele, resulting in an expansion of the expression domain of *PHB* in the stele (Vatén et al., 2011). Induction of the *cals3m* in the vasculature inhibits the movement of SHR proteins from the stele to the endodermis (**Figures 1C,D**), confirming that the *SHR* protein moves via PD and that the *icals3m* system can be used to interfere intercellular protein trafficking (Vatén et al., 2011; Sevilem et al., 2013). The ability of this system to hinder the movement of miRNAs has also been analyzed. An *in situ* hybridization analysis for GT expressed *MIR165a* (*J0571; p6xUAS::MIR165a*) shows that upon *cals3m* induction in the GT (*J0571; p6xUAS::icals3m*) of *shr* mutant, the movement of *MIR165*a to the vascular tissues can be inhibited (**Figures 2A,B**; Vatén et al., 2011). This was further validated by creating a "miRNA-sensor" system by combining *icals3m* with a modified version of *MIR165A* gene, called *MIR165Amu* that is designed to target a broadly expressed nuclear-localized YFP, nlsYFP (Miyashima et al., 2011; Vatén et al., 2011; Sevilem et al., 2013). GT-specific expression of *MIR165Amu* (*J0571; p6xUAS::MIR165Amu*) is sufficient to remove the nlsYFP signal from the stele, however, once the movement of *MIR165Amu* is inhibited by inducing the *cals3m* in the GT (*J0571; p6xUAS::icals3m*), the nlsYFP signal re-appears in the stele. These results together suggest that *icals3m* can be effectively used to inhibit trafficking of a broad range of non-cell autonomous proteins and small RNAs.

USING THE *ical3m* TO STUDY BIOLOGICAL PROCESSES RELYING ON SYMPLASTIC COMMUNICATIONS

In addition to its application for mobility analysis of proteins and miRNAs, Bishopp et al. (2011) used the system to elegantly demonstrate that cytokinins translocate from shoot to root via phloem. They applied ^{14}C-labeled cytokinin on the hypocotyls (a shoot tissue) of wild-type, *apl* mutants that lack phloem tissues and to a transgenic line expressing *icals3m* in the phloem tissues (*pAPL::XVE>>cals3m*). The fluorescence was visualized

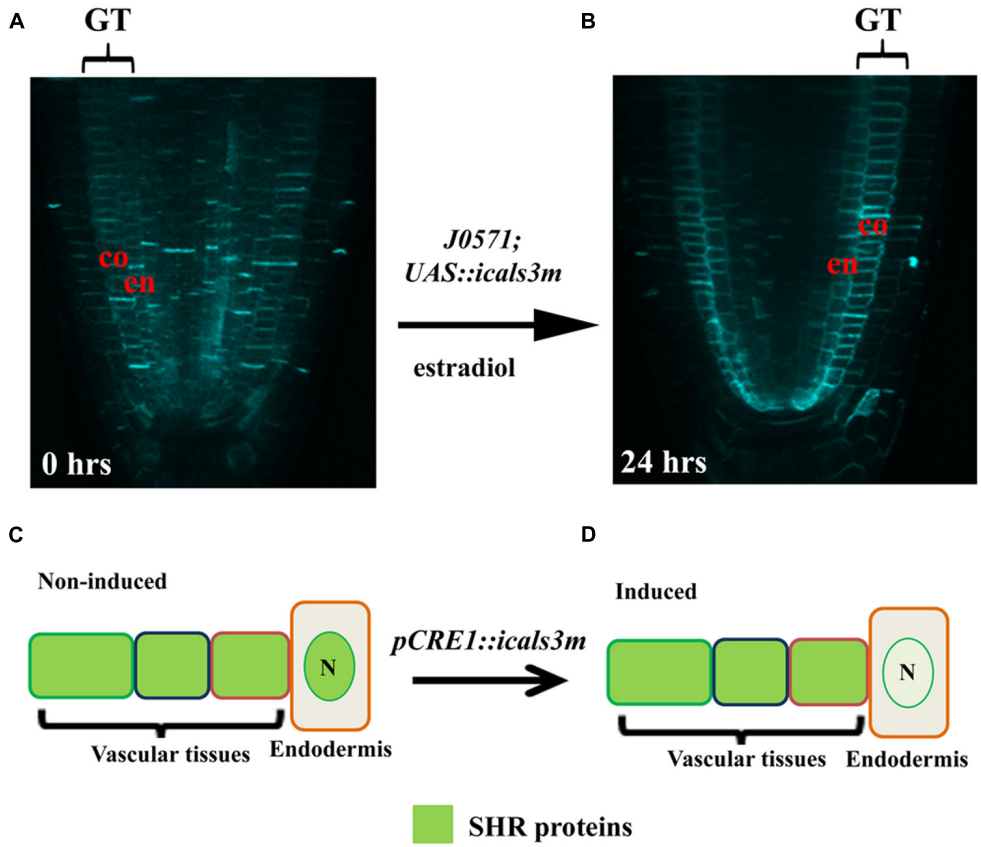

FIGURE 1 | Inducible expression of *cals3m* in ground and vascular tissues. (A, B) Aniline blue stained *Arabidopsis* root tips of the *J0571; p6xUAS::icals3m* line show that upon 24 hrs 17β-estradiol treatment, callose accumulation is enhanced in the ground tissues (GT); the endodermis (en) and the cortex (co) in **(B)**. Non-induced root does not show callose in these tissues **(A)**. **(C,D)** Schematic diagram showing the use of the *icals3m* system in studying the route of SHR protein movement. **(C)** In non-induced condition, the SHR protein (green signal) is distributed in the cytoplasm of vascular tissues but after moving to the endodermis, the protein gets localize to the nucleus (N). **(D)** When *cals3m* is induced in the vascular tissues using *CRE1* promoter, it blocks the movement of SHR protein from vascular tissues to the endodermis, suggesting that SHR protein move through PD (Vatén et al., 2011).

and the radioactive signals were quantified in the root apex to analyze long-distance transport of cytokinins. In contrast to wild-type, the basipetal transport of ^{14}C-labeled cytokinins was highly compromised in the *apl* mutants and after *cals3m* induction in the phloem of the *pAPL::XVE>>cals3m* lines (**Figures 2C,D**; Bishopp et al., 2011), suggesting that *icals3m* can also be used to hinder the long-distance transport of mobile molecules.

Moreover, apart from analyzing the mobility of a candidate molecule, the *icals3m* has also been used, as a supporting technique, in studying the significance of symplastic domain formation during LR patterning (Benitez-Alfonso et al., 2013). When *cals3m* was induced in the LR-competent xylem pole pericycle (XPP) cells using an enhancer trap-line, *J0121* (*J0121>>cals3m*), both the LR density and positioning were affected, supporting the hypothesis that controlled intercellular symplastic connectivity among pericycle cells, founder cells and the neighboring tissues is important for *Arabidopsis* LR patterning (Benitez-Alfonso et al., 2013). This study provides an additional value to *icals3m* system that it can be applicable in interfering symplastic domain formation during organ development.

FUTURE PERSPECTIVES

In addition to large signaling molecules (e.g., proteins and RNAs), small molecules such as nutrients and hormones also move through PD. A recent quantification of PD flux in the root meristem demonstrates that the PD flux is actually 10-fold higher than reported in an earlier study (Goodwin et al., 1990; Rutschow et al., 2011), suggesting that an efficient symplastic diffusion may be a major route for the transport of nutrients in the meristem. Interestingly, the solute flux is reduced in *Arabidopsis* line overexpressing *PDCB1*, a protein that promotes callose deposition at PD (Rutschow et al., 2011), indicating that enhanced callose deposition at PD can inhibit solute movement. Therefore, *icals3m* might be equally applicable for obstructing the symplastic movement of metabolites through PD.

In summary, PD-mediated symplastic communication provides a major route for the movement of positional signals during plant development, and callose turnover at PD confers an important mechanism to regulate symplastic trafficking. The *icals3m* system is an effective tool to hinder symplastic trafficking through PD in a spatially and temporally regulated manner. This system

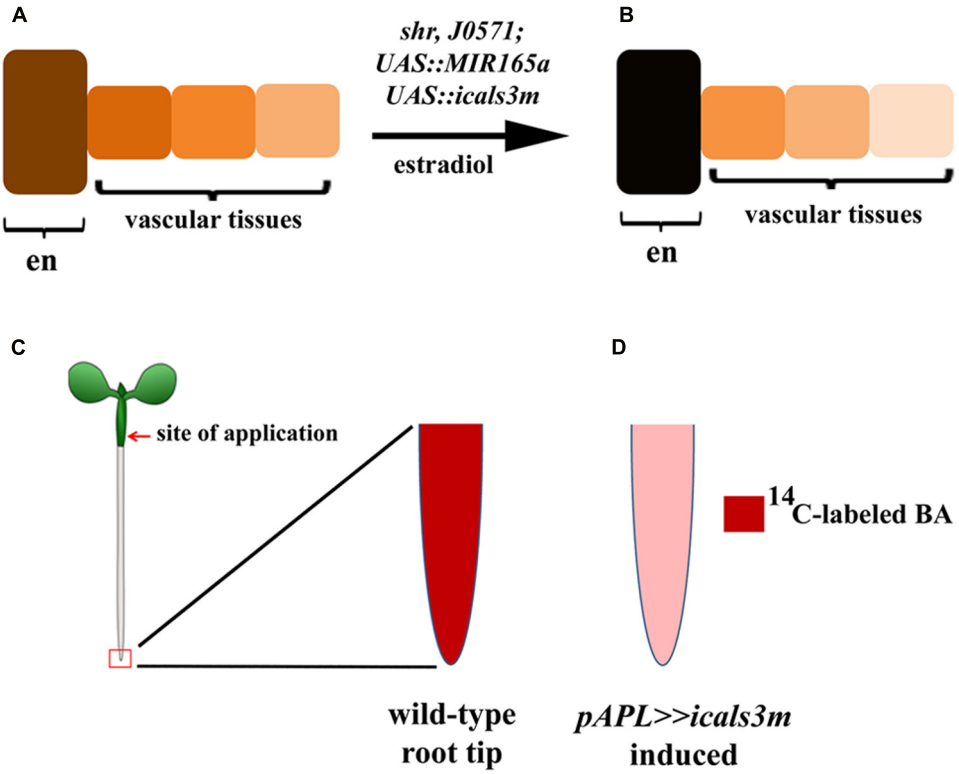

FIGURE 2 | Applications of the *icals3m* system in studying the route of miR165 and long-distance transport of cytokinins. (A,B) Schematic diagram illustrating the use of *icals3m* in inhibiting the movement of miR165 from the endodermis to the vascular tissues. Only endodermis (en) of GT is shown in the schematic figure. **(A)** Under non-inducible condition, the GT expressed miR165a can diffuse freely from endodermis to the vascular tissues and makes a concentration gradient. The cells directly connected to the endodermis have high levels of miRNAs whereas miRNA levels decrease in the cells that are away from the endodermis, as shown by color gradients. (B) Upon *cals3m* induction in the GT, the miRNAs are getting trapped inside the endodermis and their concentrations in the vascular tissues are reduced. The color codes highlight miRNA concentrations in the endodermis and the vascular tissues, before **(A)** and after induction **(B)**. The miRNA signals in the endodermis of estradiol-treated plants are higher than the mock-treated plants whereas opposite pattern is seen in the vascular tissues. **(C,D)** The utility of *icals3m* in analyzing the shoot-to-root transport of cytokinins in *Arabidopsis*. ^{14}C-labeled N6-benzyladenine (BA) was applied to the hypocotyls (shown by red arrow) and accumulation of the radio labeled isotope was analyzed in the root tips (highlighted by red box). **(C)** In wild-type roots, a high level of radioactive signal is seen in the root tip which is significantly reduced upon blocking the symplastic connectivity in the phloem tissues by expressing *icals3m* under *APL* promoter (**D**; Bishopp et al., 2011).

has been successfully applied to inhibit movement of proteins, miRNAs, cytokinin, and to interfere a symplastic domain formation during LR development. Therefore, we envision that it can also be efficiently used for inhibiting the symplastic transport of nutrients and metabolites. Moreover, the *icals3m* system could be used widely, in combination with other approaches, to investigate various molecular events relying on symplastic signaling.

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Refurbishing the plasmodesmal chamber: a role for lipid bodies?

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Lipid bodies (LBs) are universal constituents of both animal and plant cells. They are produced by specialized membrane domains at the tubular endoplasmic reticulum (ER), and consist of a core of neutral lipids and a surrounding monolayer of phospholipid with embedded amphipathic proteins. Although originally regarded as simple depots for lipids, they have recently emerged as organelles that interact with other cellular constituents, exchanging lipids, proteins and signaling molecules, and shuttling them between various intracellular destinations, including the plasmamembrane (PM). Recent data showed that in plants LBs can deliver a subset of 1,3-β-glucanases to the plasmodesmal (PD) channel. We hypothesize that this may represent a more general mechanism, which complements the delivery of glycosylphosphatidylinositol (GPI)-anchored proteins to the PD exterior via the secretory pathway. We propose that LBs may contribute to the maintenance of the PD chamber and the delivery of regulatory molecules as well as proteins destined for transport to adjacent cells. In addition, we speculate that LBs deliver their cargo through interaction with membrane domains in the cytofacial side of the PM.

Keywords: hemi-fusion, lipid droplet, 1,3-β-glucanase, membrane raft, microdomain, oleosin, shoot apical meristem, SNARE

INTRODUCTION

Recent progress in isolation procedures and proteomic approaches expanded the protein inventory of a generalized plasmodesma (PD), but despite this the PD-proteome is still largely elusive (Bayer et al., 2006; Fernandez-Calvino et al., 2011; Jo et al., 2011). The effort to understand PD functioning from PD composition is faced with several obstacles.

Firstly, PD differ widely among the different cells, tissues and organs of a plant. The main reason for this diversity is the way higher plants growth and development, how they build their body and allocate functions to various parts. Their entire shoot system is derived from the shoot apical meristem (SAM). Daughter cells, produced in cell lineages at the SAM, remain connected via primary PD that are laid down in cell plates. To maintain the necessary symplasmic unity, adjacent lineages become connected via secondarily formed PD. These two distinct mechanisms of PD initiation define the original composition, architecture and function of so-called primary and secondary PD (Rinne and van der Schoot, 1998; van der Schoot and Rinne, 1999). When cells embark on a path to differentiation and specialization, PD structure and function are altered further in correspondence to their position. Thus, rather than being unit structures, PD reflect the functional states of the interconnected cells.

Secondly, the highly dynamic nature of PD in general, but particularly in meristems and developing tissues, might preclude unambiguous establishment of a PD proteome even in a single tissue system. It might turn out that the PD proteome is inherently contingent, and many proteins that associate with PD might be only temporary constituents and regulators, or simply passers-by.

Thirdly, PD do not function in isolation and their proteome is intimately dependent on the regulation of distinct supply routes that deliver components to the exterior and interior of PD. Thus, understanding PD functioning in addition requires identification of the pathways by which proteins are recruited to the exterior and interior of PD, and the mechanisms by which they cooperatively govern PD dynamics. So far, very little is known about these supply routes and how they are coordinated.

Although PD composition and functioning is most conveniently investigated in the large cells of differentiated tissues, PD functioning is likely to be most versatile and sophisticated in meristematic areas, where morphogenetic signaling is expectably very intense. For several reasons therefore, meristems are of prime interest for the investigation of PD structure and function. Despite their minute size, shoot apices of perennials provide a unique and unexpected experimental opportunity to study PD that cyclically change their structure and function in synchrony with the seasons. Anticipating winter, the SAM of deciduous perennials arrests itself in a morphogenetically deactivated and dormant state. This state is enforced by the production of dormancy sphincter complexes (DSCs). DSCs function as symplasmic circuit breakers that hermetically close all PD by a precise deposition of a callosic mixture around the PD entrance and inside the channel (Rinne et al., 2001). Simultaneously, the isolated cells amass minute lipid bodies (LBs) with a coat of proteins. Associated with the LB surface is a subset of 1,3-β-glucanase (GH17-family) enzymes (**Figure 1**). During chilling-induced release from dormancy these LBs target the plasmamembrane (PM) at, or in close proximity to PD, thereby facilitating restoration of PD functionality (Rinne et al., 2011).

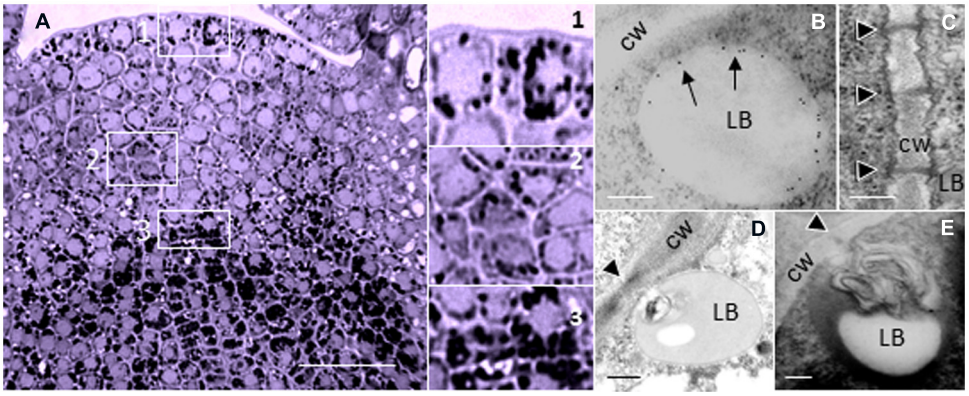

FIGURE 1 | Lipid bodies in the perennial shoot apex. (A) Shoot apex of hybrid aspen (*Populus tremula* × *P. tremuloides*) after exposure to short photoperiod results in the accumulation of LBs during initiation of dormancy. LBs are visualized by Sudan Black B. Boxed areas (1–3) are detailed on the right; 1 = Tunica, 2 = Corpus, 3 = Rib meristem/Rib zone. **(B–E)** Electron microscopic images of LBs in shoot meristem of birch (*Betula pubescens*) during chilling-induced release from dormancy. **(B)** Immuno-gold-labeling of 1,3-β-glucanase, peripherally associated with a LB. Arrows point to gold particles that label 1,3-β-glucanase (Form Rinne and van der Schoot, 2004). **(C)** PD in the cell wall of after removal of callosic dormancy sphincter complexes (From Rinne et al., 2001). **(D,E)** Membranous inclusions, probably desmotubule-attached cortical ER in LB that dock PD. Monolayer membranes of LBs are visualized by Osmium tetroxide and tannic acid. Black arrowheads point to PD (From Rinne and van der Schoot, 2004). Bars, **(A)** 50 mm; **(B–E)** 250 nm.

In multicellular organisms LBs have emerged as signaling platforms that deliver proteins and signaling molecules to a variety of intracellular destinations (Murphy, 2012). It seems possible that in plants, LBs have assumed the additional function of a vehicle that delivers proteins to PD for cell-to-cell transport, or regulation and refurbishment of the PD interior (van der Schoot et al., 2011). If so, it would be opportune to analyze the LB proteome and investigate to what degree it overlaps with the PD proteome. In a morphogenetically active SAM, the amount of LBs is far too restricted to make them amenable to biochemical analysis. Fortunately, the dormant apex offers a unique opportunity to isolate sufficient amounts of LBs to analyze their proteome and test this hypothesis. As most of our novel knowledge on LB composition and function is derived from animal systems, we first review crucial findings from the animal literature before we address the question if in plants LBs may contribute to refurbishing the PD interior.

ORIGIN OF LIPID BODIES

LBs, often called lipid droplets, are of universal occurrence, and have been observed for over a century (Murphy, 2012). In contrast to what the latter name suggests, they are not simple droplets. On the contrary, they are minute membrane-bound organelles, ranging in size from about 0.5 to 2.5 μm, which are produced by specialized areas of the tubular endoplasmic reticulum (ER; **Figure 2**). It is increasingly clear that they are heterogeneous and dynamic entities that serve important regulatory functions.

LBs possess a core of neutral lipids, triglycerides (TAGs) or sterol esters, and a surrounding phospholipid (PL) monolayer (Chapman et al., 2012; Murphy, 2012). This is a relatively stable configuration, with PL acyl-moieties in the hydrophobic core and the charged headgroups in the cytoplasm. TAGs are synthesized in the ER (Huang, 1996) and deposited between the leaflets of the ER membrane. The highly hydrophobic TAGs have low membrane solubility and will "oil out" between the leaflets, forming a lens-like structure (Olofsson et al., 2009). That LBs originate from the ER membrane is supported by their similar PL composition (Fujimoto et al., 2008). However, the detailed mechanism of LB formation has remained elusive, and different models have been proposed (Guo et al., 2009; Walther and Farese, 2009).

Most frequently LB formation is described in terms of a "bulging and budding" model. It depicts LB biogenesis as a process in which nascent LBs bud off from the cytoplasmic side of the ER. As a consequence, the LB monolayer is exclusively derived from the cytoplasmic leaflet of the ER (**Figure 2**). In the "bicelle" or "hatching" model (Ploegh, 2007; Fujimoto et al., 2008), the complete oil lens is cut off from the ER, resulting in a LB monolayer that contains parts of both leaflets. In a third model, the "vesicular budding" model, minute bilayer vesicles are formed that stay tethered to the cytoplasmic side of the ER while a shuttle mechanism transports neutral lipids into its bilayer. This results in a growing LB with a minuscule inner aqueous inclusion that is surrounded by the original luminal leaflet of the ER membrane. This model might imply involvement of coatamers, which assist vesicle formation in the secretory pathway, as knockdown of the COP1/Arf1 machinery interferes with LB formation (Guo et al., 2008). It is possible, however, that all these mechanisms are valid and operate alongside each other. The composition of the protein coat is strongly dependent upon the LB production mechanism, which determines whether only the proteins of the cytoplasmic leaflet of the ER are included or also those at the luminal ER leaflet.

PROTEIN COMPOSITION OF THE NASCENT LB COAT

The particular structure of a LB restricts what kind of proteins can associate with it. The normal configuration of transmembrane (TM) proteins, with the hydrophilic domains on opposite

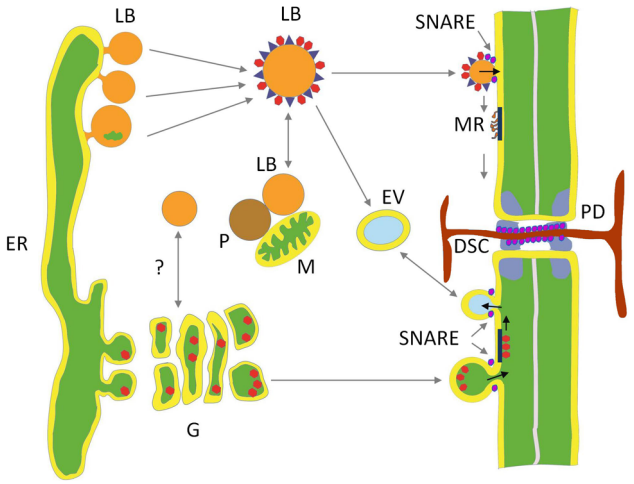

FIGURE 2 | Hypothetical model depicting two delivery paths to plasmodesmata. Lipid bodies (LBs) deliver cargo to membrane rafts (MR) at the inner leaflet of the plasma membrane (PM) and the plasmodesmal (PD) channel. LBs are pinched off from specialized areas of tubular ER. Their core of neutral lipids is covered by a protein coat composed of structural proteins, enriched by proteins donated by interacting organelles, like mitochondria (M), peroxisomes (P), early endosome vesicles (EV), and possibly Golgi (G) vesicles. Some of the LBs target PM and transfer proteins to MR that transport them to the PD cavity. In the dormant perennial shoot apical meristem one of the LB proteins is a peripherally associated 1,3-β-glucanase that hydrolyzes the callose plug of the dormancy sphincter complex (DSC). The secretory path delivers cargo that is packed in ER-derived vesicles, among which glycosylphosphatidylinositol (GPI)-anchored 1,3-β-glucanases, that are moved through the Golgi to fuse as exocytic vesicles with the PM, releasing their cargo to the outer side of the PM, where selected GPI-anchored proteins are potentially recruited by MRs for transport to the outer leaflet of the PD neck. Soluble NSF attachment protein receptors (SNAREs) mediate endocytosis, exocytosis, and presumably hemi-fusion of LBs with the PM.

sides of the membrane, is not feasible in the LB monolayer due to the hydrophobic core. Instead, constitutive LB proteins possess a long hydrophobic domain that forms a hairpin-helix which anchors the protein to the lipid core, while the hydrophilic termini are spread out at the LB surface. Examples of LB proteins with such hairpin topology are caveolin (Martin and Parton, 2006) and the TG- and cholesterol-catalyzing ER enzymes DGAT2 and NSDHL in mammalian cells (Caldas and Herman, 2003; Stone et al., 2006; Murphy et al., 2009), and oleosin and caleosin in plant cells (Lee et al., 1994; Huang, 1996; Chapman et al., 2012; Murphy, 2012).

Alternatively, proteins associate with a LB by embedding amphipathic domains into the monolayer (Brasaemle, 2007; Fujimoto et al., 2008; Guo et al., 2009, Walther and Farese, 2009). Mammalian examples are PAT proteins (including perilipin, adipocyte-differentiation-related-protein [ADRP], and tail interacting protein 47 [TIP47]). They are recruited post-translationally, and are either exclusive for LBs or present in the cytoplasm as well as at LBs. For example, ADRP and perilipin are constitutively associated with LBs via hydrophobic domains, and in absence of neutral lipids they are degraded. In contrast, TIP47 is a soluble cytosolic protein with a terminal hydrophobic domain which is recruited to LBs under elevated fatty acid levels (Wolins et al., 2001), possibly requiring a change in the shape of its hydrophobic pocket (Fujimoto et al., 2008).

An important group of LB-associated proteins are Rab GTPases, which are involved in membrane sorting and targeting (Grosshans et al., 2006; Bartz et al., 2007; Liu et al., 2007). They function as molecular switches which in their active GTP-bound forms recruit effector proteins to mediate vesicle motility, docking, and fusion (Jordens et al., 2005; Liu et al., 2007).

KISS-AND-RUN ENCOUNTERS AND REFUGEE PROTEINS

Structural studies indicate that LBs interact with other organelles (Binns et al., 2006; Shaw et al., 2008). This is supported by proteomic studies of LBs, revealing the presence of proteins that are characteristic of mitochondria, peroxisomes, endosomes, ER, and PM (**Figure 2**; Goodman, 2008; Guo et al., 2009; Murphy et al., 2009). Transient interactions, mediated by small GTPases, allow the exchange of lipophilic signals and proteins that are embedded in the monolayer or electrostatically attached to its surface (Liu et al., 2008).

That LBs functionally dock to mitochondria (Shaw et al., 2008) is supported for example by fluorescence resonance energy transfer, which provides evidence that their membranes are in direct physical contact (Sturmey et al., 2006; Zehmer et al., 2009). LBs also interact with peroxisomes, to deliver lipids for β–oxidation (Binns et al., 2006; Zehmer et al., 2009) and recruit Rab5 and Rab11 to interact with endosomes (Frolov et al., 2000; Liu et al., 2007). Lipid exchange might proceed in an ATP-independent fashion, as proposed for PM–ER contact sites in yeast (Schnabl et al., 2005), involving transient-inter-compartmental-contact-sites (TICCS; Liu et al., 2007; Zehmer et al., 2009). Alternatively, docking events may be followed by hemi-fusion of the LB monolayer with the outer leaflet of a bilayer structure (Murphy et al., 2009). Cytoplasmic LBs may even usurp ER (Zehmer et al., 2009) as whorls of ER, ribosome-decorated ER, and RNA were detected in the lipid core of some LBs (McGookey and Anderson, 1983; Wan et al., 2007; Zehmer et al., 2009).

Some LBs show Brownian movement, as if waiting for delivery orders, while others move in a coordinated and directional fashion. In animal cells, LBs move on microtubules with dynein motor proteins, but as actin and myosin are also present in the LB proteome they might have ancillary roles (Turró et al., 2006; Bartz et al., 2007; Welte, 2009). In contrast, in plants actin is the major organelle transporter, while microtubules have an assisting role (Collings et al., 2002; Cai and Cresti, 2012). Virtually all encounters between LBs and other organelles are of a transient "kiss-and-run" fashion (van Manen et al., 2005). En route along the cytoskeletal highway, LBs may also pick up proteins and signaling molecules that opportunistically hitch a ride to their destination. Relatively hydrophobic proteins that do not move easily through the aqueous environment of the cytoplasm might piggyback on the lipid shuttle (Welte, 2009). These accidental travelers have been referred to as "refugee proteins" (Hodges and Wu, 2010).

As a direct result of these frequent kiss-and-run encounters and the boarding of opportunistic passengers the LB proteome is surprisingly rich (Welte, 2009; Hodges and Wu, 2010). Proteomic studies of mammalian LBs show that they contain dozens, and

perhaps hundreds of proteins (Bartz et al., 2007; Zehmer et al., 2009). For example, a recent investigation identified 125 LB proteins, including Arf1, Arf1 binding protein, coatamers of Arf-1, small G-proteins, lipid synthetic enzymes, chaperones (HSPs), vimentin, calreticulin-3, calnexin, spectrin, heavy-chain myosin, actins, and tubulins (Bartz et al., 2007). As pointed out, the large number of Rabs in these LBs, 18 in total, might indicate that there are distinct classes of LBs with corresponding composition and intracellular destinations (Bartz et al., 2007). Supportive of the validity of such LB inventories is the finding that RNAi screens identified hundreds of genes that are involved in LB biology (Beller et al., 2008; Guo et al., 2008).

Due to the virtual absence of extensive LB-proteome inventories in plants, the number of identified LB-associated proteins is still low. However, there is no a priori reason to expect that the situation in plants is much different from that in animals. The number of peripherally associated LB-proteins, particularly enzymes and signaling molecules, might be equally large. So far, the inventory of proteins found at plant LBs includes among others the structural proteins oleosin and caleosin (Sarmiento et al., 1997; Tzen et al., 1997; Næsted et al., 2000), which both appear to possess enzyme activities (Hanano et al., 2006; Meesapyodsuk and Qiu, 2011; Parthibane et al., 2012), the stress-inducible caleosin RD20 (Aubert et al., 2010), the sterol-dehydrogenase steroleosin (Lin et al., 2002), a peroxygenase (Hanano et al., 2006), a hydroxysteroid dehydrogenase (Li et al., 2007), a lipoxygenase (Hause et al., 2000), an acid lipase (Eastmond, 2004), a patatin-domain lipase (May et al., 2000; Eastmond, 2006), several non-glycosylphosphatidylinositol (GPI)-anchored 1,3-β-glucanases (**Figure 1B**; Rinne et al., 2001, 2011; Rinne and van der Schoot, 2004), the innate immune-response protein calcium-dependent kinase CPK1 (Coca and San Segundo, 2010), glyoxisome receptors (Hause et al., 2000), and various unidentified proteins (Tnani et al., 2011). Many other LB-associated proteins in animal cells have homologs in plants where they may similarly associate with LBs.

PLANT LBs DELIVER CARGO TO PD

LBs potentially deliver proteins and other associated components to the PD interior in two ways. Firstly, LBs may directly interact with PD and with the cortical ER strands. Transmission electron microscopy showed that during chilling-induced release from dormancy, LB are displaced from random cytoplasmic positions to the PD (Rinne et al., 2001; Rinne and van der Schoot, 2004) where they can usurp membranous material, possibly from ER strands that are continuous with the desmotubule in the center of the PD channel (**Figures 1D,E**). How these LBs deliver the peripherally associated 1,3-β-glucanases (GH17 family proteins) to the callose deposits at the PD channels is uncertain. Secondly, overexpression of eGFP-tagged LB-associated GH17 proteins appeared to target the PM and PD in leaf cells (Rinne et al., 2011). Whereas GPI-anchored eGFP-tagged GH17 proteins labeled PD in punctate patterns, the LB-associated GH17 proteins mostly localized at the PM in distinct sandwich-like patches that are indicative of delivery into some kind of PM domains (Rinne et al., 2011). GPI-anchored proteins are produced in the ER and after post-transcriptional modification send through the Golgi system to the cell's exterior, where they are anchored to microdomains at the extrafacial leaflets of the PM (see below). It seems possible that, in contrast, LB-associated GH17 proteins and other LB-associated cargo are recruited to membrane rafts (MRs) or microdomains at the cytofacial side of the PM (Rinne et al., 2011). This would require a functional relation or organizational similarity between MRs or microdomains and LBs.

MRs are considered special nano- or microdomains that are composed of sphingoplids, esters and proteins (Simons and Toomre, 2000; Lingwood and Simons, 2010). Interestingly, in adipocytes, the LB-monolayer is covered by unesterified cholesterol (Prattes et al., 2000) and raft-associated signaling proteins like mitogen-activated protein (Yu et al., 1998, 2000) as well as the raft-associated scaffolding protein caveolin-2 (Fujimoto et al., 2001). This prompted Fujimoto et al. (2001) to speculate that LBs function as a novel membrane domain, with caveolin residing in raft-like domains. This "sensational proposal" (van Meer, 2001) warrants a closer look.

MEMBRANE RAFTS AND DOMAINS

It is well-established that lipid-based rafts in the PM are ordered domains of sterols and highly saturated sphingolipids that arise by self-association within a more disordered environment (Simons and Toomre, 2000; Jacobson et al., 2007). These domains, referred to as lipid rafts (LRs; Simons and Toomre, 2000; Rajendran and Simons, 2005) or MRs (Langlet et al., 2000) were originally conceived in terms of the liquid-ordered (Lo) and liquid disordered (Ld) phases found in purified lipid systems. These model systems did not give a realistic picture of MRs in the PM as they also contain selected TM proteins that are excluded from the Lo phase when reconstituted in a model system (Lingwood and Simons, 2010). Isolation of detergent-insoluble (or resistant) membrane fractions (DIMs or DRMs) yielded a large number of PM proteins that seemed to be part of MRs. In plants such fractions could for example contain leucine-rich-repeat (LRR) as well as other receptor-like kinases (RLKs; Peskan et al., 2000; Shahollari et al., 2004; Lefebvre et al., 2007, 2010) that are implicated in endocytosis and signaling (Duncan et al., 2002; Lingwood and Simons, 2010). However, it appeared that DIMs could not be equated with MRs, and the DIM/DRM-based raft concept has been scrutinized lately (discussed in Tanner et al., 2011). Nonetheless, the existence of PM MRs is not in dispute, and their spatio-dynamic features can be mapped by CSLM, immunochemistry and ultrastructural studies (Berchtold and Walther, 2009; Raffaele et al., 2009; Keinath et al., 2010; Mongrand et al., 2010). The current consensus is that MRs are dynamic nano-scale domains, enriched in cholesterol, sphingolipids and GPI-anchored proteins, which act as membrane-organizing "principles" (Lingwood and Simons, 2010). Nano-sized MRs can be triggered to cluster into larger microdomains by lipid–lipid, protein–protein and lipid–protein interactions (Lingwood and Simons, 2010). Although the PM of plants might differ from that in animal systems in terms of lipid composition, similar organizational principles are likely to apply, with MRs serving comparable regulatory and signaling functions (Mongrand et al., 2010; Jarsch and Ott, 2011; Perraki et al., 2012).

Universally, GPI-anchored proteins are exported via the secretory pathway and segregated into exoplasmic MRs, whereas doubly

acylated proteins are recruited by inner leaflet MRs (Simons and Toomre, 2000). The cytofacial MRs are of interest in relation to LBs as these microdomains are thought to function as signaling and docking domains. (**Figure 2**; Mongrand et al., 2010). Recently, remorins a family of plant-specific proteins were identified. Members of one group associate specifically with MRs in a sterol-dependent fashion at the inner PM leaflet, despite their overall hydrophilic nature (Raffaele et al., 2009; Jarsch and Ott, 2011; Perraki et al., 2012). In potato, REMORIN1 (StREM1.3) appears to possess a C-terminal lipid anchor, RemCA, which tethers it into the MRs (Perraki et al., 2012). Remorins are suggested to be scaffolding proteins that participate in the regulation of signaling processes by recruiting PM- and cytoplasmically located proteins into microdomains to preassemble signaling complexes (Jarsch and Ott, 2011). These may include RLKs (Lefebvre et al., 2010). Plant-specific sterols and sphingolipids in MRs can also recruit specific signaling proteins, including RLKs, G-proteins, and stress response- and dynamin-related proteins, as well as 14-3-3 proteins (Stanislas et al., 2009; Mongrand et al., 2010).

DO LBs CONTAIN RAFT-LIKE DOMAINS?

For mammalian systems the original suggestion of Fujimoto et al. (2001) that LBs may represent a new "membrane domain" seems supported by a number of findings.

For example, the scaffolding protein caveolin-2 of PM rafts can shuttle to LBs in an identical orientation, with its long central hydrophobic helix embedded in the monolayer and both hydrophilic termini in the cytoplasm; significantly it is sequestered in small clusters at the LB monolayer in domains not dissimilar to the MRs in the PM, and it can also shuttle from the ER to LBs as well as to the PM (Das et al., 1999; Ostermeyer et al., 2001, 2004; Brasaemle et al., 2004; Martin and Parton, 2006; Rajendran et al., 2007).

Notably, two PAT family proteins, adipophilin and TIP47, are present at the PM as well as at LBs. Under normal conditions they are dispersed in the PM of macrophages and adipocytes, but stimulation of LB formation by incubation with acetylated low density lipoprotein induces their aggregation in elevated PM domains (Robenek et al., 2009). Although much larger than MRs, roughly 1.0–1.5 μM in diameter, these areas clearly represent membrane domains. That LBs are closely apposed to these elevated PM domains seems remarkable. Cytoplasmically localized TIP47 can associate with LBs by changing its hydrophobic pocket (Fujimoto et al., 2008), and this may also underlie its association with the elevated PM domains. Interestingly, in plants the potato remorin StREM1.3 similarly associates with PM rafts or microdomains by changing the configuration of the short C-terminal anchor RemCA. In the cytoplasm the anchor is unordered but in a non-polar lipid environment it spontaneously folds into a hairpin structure with amphipathic-helices that is inserted into the PM (Perraki et al., 2012).

In addition, LBs can contain flotillin-1, which is regarded as a true MR marker (Babuke and Tikkanen, 2007). Flotillin-1 and flotillin-2 associate with the MR in the PM through acylation sites (Neumann-Giesen et al., 2004; Morrow and Parton, 2005; Otto and Nichols, 2011), as well as through the prohibition homology domain (PHB) which has a putative hairpin-like topology, similar to that of caveolins (Bauer and Pelkmans, 2006). Flotillin-1 and -2 co-assemble into stable, yet mobile complexes at the PM that act as scaffolds, demarcation sites for targeted cargo delivery (Stuermer, 2011), and signaling platforms (Ludwig et al., 2010). They can also function as sensors that detect changes in membrane tension (Ge et al., 2011) and may guide the budding of MRs to emerging LBs (Neumann-Giesen et al., 2004; Rajendran et al., 2007). Interestingly, flotillins are also present in plants at the PM, and are required for entry of nitrogen-fixing bacteria (Haney and Long, 2010). Similarly, MR-associated remorin of *Medicago truncatula* (MtSYREM1) is specifically induced during root nodulation and it accumulates at rhizobia release sites (Lefebvre et al., 2010) that were earlier characterized by presence of the syntaxin SYP132 (Catalano et al., 2007). Based on this evidence, it is tempting to speculate that in plant cells flotillins and syntaxins (see below) may associate with LBs that align with remorin-decorated MRs.

Interestingly, the oligomeric protein stomatin (Stom), a PM raft-associated integral protein, localizes to the late endosomal compartment, and when overexpressed also to LBs (Umlauf et al., 2004). Live microscopy showed that StomGFP-tagged LBs interact with multiple microtubule-associated vesicles, and that stomatin and caveolin-3 may localize to distinct domains at the LB surface (Umlauf et al., 2004). Stomatin has a topology that enables it to associate with rafts as well as LBs. Its C-terminal domain is necessary for raft formation, whereas the long hydrophobic domain tethers it to LBs (Umlauf et al., 2004), much alike a similar hairpin in caveolin-1 (Bauer and Pelkmans, 2006).

The above examples show that there is a relation between LBs and PM micro domains in both animal and plant systems, although the precise nature of that relation is unclear. In plants, the LB monolayer may not contain cholesterol, and therefore the monolayer might not count as a genuine MR, that is, as LR with associated proteins. This does not preclude interaction or exchange, as proteins could have separate domains for targeting LBs and PM rafts, as in case of stomatin. It seems reasonable to propose that LBs represent some kind of "membrane domain," the more so, as cholesterol might not always be a prerequisite for domain formation. Recently it was shown that electrostatic protein–lipid interactions can give rise to microdomains independently from cholesterol or lipid phases (van den Bogaart et al., 2011). In any case, the examples lend support to the notion that LBs in some way interact with MRs or microdomains to deliver or exchange proteins and lipids. Interestingly, LBs in animal systems are known to contain a number of soluble NSF attachment protein receptors (SNAREs) that are involved in LB fusion. For example, the SNARE syntaxin5 anchors itself in the lipid core, SNAP23 in the LB monolayer, whereas VAMPP4 associates with the LB surface (Boström et al., 2007; Olofsson et al., 2009; Zehmer et al., 2009). It seems likely that LBs can also undergo hemi-fusion with PL bilayers, such as the PM, permitting the transfer of peripherally associated proteins, such as caveolin (Murphy et al., 2009). Hemi-fusions are in terms of energy expenditure less costly than a bilayer fusion, and easier to perform (Murphy et al., 2009). SNARE- and Rab-assisted transient hemi-fusions between LBs and PM domains could possible explain why some MR proteins can be transferred to LBs and vice versa. For

example, interaction of LBs and PM caveolae may allow a transient hemi-fusion for the exchange of the MR protein caveolin (Murphy et al., 2009). Significantly, at the plant PM SNAREs might be distributed in microdomains to mediate exocytosis of secretory vesicles (Sutter et al., 2006), and it is tempting to speculate that LBs might hemi-fuse with the PM at mobile microdomains (**Figure 2**).

REFURBISHING THE PD INTERIOR: A ROLE FOR LBs?

In general, three pathways could be envisioned through which proteins and other components reach the PD exterior and interior. A pathway that delivers proteins to the PD exterior is the secretory pathway through which GPI-anchored proteins, produced in the ER and modified in the Golgi, reach the cell wall and the PD. GPI-anchored proteins are delivered together with sterols and sphingolipids to the cell exterior, like in animal cells. At the exofacial leaflet of the PM they are anchored to MRs, the assembly of which starts in the Trans Golgi Network (Varma and Mayor, 1998; Lingwood and Simons, 2010). In plants, some of these secreted GPI-anchored proteins are recruited to the exterior of PD. Although these proteins might be released to the outside of the PM in close proximity of PD (Oparka, 2004), they have to move laterally to reach the PD neck (Tilsner et al., 2011). As in animal cells MRs are considered to be relatively mobile platforms, it seems reasonable to assume that in plant cells the MRs can move their resident proteins through lateral displacement to PD. Several recently identified proteins could reach the PD exterior this way. For example, GPI-anchored 1,3-β-glucanases (GH17 family proteins) are exported and transferred to the PD neck, where they hydrolyse callose (Levy et al., 2007; Rinne et al., 2011). The GPI-anchored PD-callose-binding protein (PDCB1), which possesses the carbohydrate binding module family 43 that is also found in a number of GH17 proteins (Rinne et al., 2011), is similarly secreted and transferred to the PD neck to link the PD membrane to the callose deposits in the external sphincter ring (Simpson et al., 2009). Another example is plasmodesmata located protein1a (PDLP1a), one of the eight members of the RLK family PDLP1, which reaches PD via the Brefeldin A-sensitive secretory pathway (Thomas et al., 2008). PDLP1a lacks a GPI domain and instead possesses a 21 amino acid transmembrane domain (TMD) that is necessary and sufficient to target PD (Thomas et al., 2008). The TMD is suggested to contain a sorting signal that interacts with other TM proteins during recruitment into a microdomain (Thomas et al., 2008) and may reach the PD through lateral diffusion in the PM (Tilsner et al., 2011). Several other TMD-containing RLKs, with putative roles in stress response pathways, are also localized to PD (Jo et al., 2011).

The other two pathways could deliver proteins to the PD channel, either via the PM or via the ER. Non-secreted proteins could be collected at PD from the cytoplasm via microdomains in the cytofacial leaflet of the PM, either after direct recruitment by scaffolding proteins such as remorin, or after delivery to such scaffold-microdomain clusters by LBs. Delivery of LB cargo is by definition to the PD channel, as the different topologies of the PM double layer and the LB monolayer prevent delivery to the outside of the cell. LB proteins destined for the PD channel could be either permanent residents or only temporary visitors and passers-by to a destination in the adjacent cell. LB routing might be guided by the actin cytoskeleton, as suggested elsewhere (Rinne and van der Schoot, 2004). In case LBs would undergo hemi-fusion with the PM, this would result in lateral diffusion of neutral lipids from the LB core into the PM, and recruitment of cargo to microdomains. Proteins that are peripherally associated with LBs could also peripherally associate with such microdomains, either

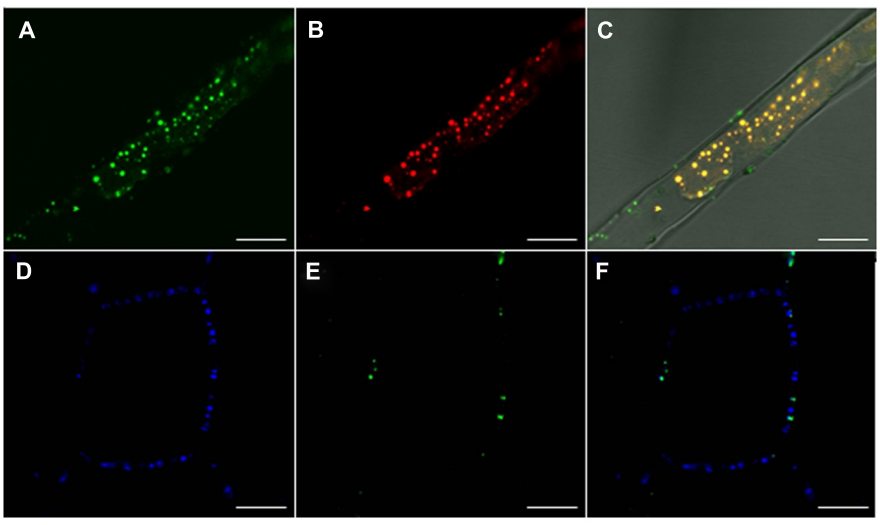

FIGURE 3 | Lipid body oleosin localizes at the plasma membrane and co-localizes with callose at plasmodesmata. (A–C) Confocal images of *Arabidopsis* root hairs in GFP::Ole2 lines showing **(A)** LB marker-protein, oleosin, GFP::Ole2, **(B)** in connection with lipid after staining with fluorescent dye Nile red. **(C)** Overlay with bright field shows that GFP::Ole2 co-localizes with LBs, except at the plasmamembrane, where GFP::Ole2 containing bodies are free of lipid. **(D–F)** Confocal images of leaf cells of *Arabidopsis* GFP::Ole2 lines showing PD-callose stained with aniline blue **(D)**, and the LB marker-protein GFP::Ole2 **(E)**. **(F)** Overlay shows that GFP::Ole2 has low expression in leaf cells, but co-localizes with callose at PD. Bars, 10 mm.

by embedding amphipatic domains in the cytofacial leaflet or by electrostatic interactions. If so, PM microdomains might shuttle a diverse cargo of LB-delivered non-integral membrane proteins. Most of these proteins might hitch a ride on the LB surface to reach the PD channel for cell-to-cell transport. Alternatively, structural proteins might become embedded in the architectural fabric of the PD channel, a specialized membrane adhesion site (**Figures 2 and 3**; reviewed in Tilsner et al., 2011). Remorin, which accumulates in the PD channel recruits PM- and cytoplasmic proteins into signaling complexes, and there seems no reason why remorin or as yet unidentified scaffolding proteins could not mediate transfer of LB-delivered proteins to the PD chamber. Regardless the precise mechanism, recent investigations showed that LB-associated 1,3-β-glucanase (Rinne et al., 2011) as well as the LB marker oleosin:eGFP target the PM and accumulate at PD (**Figure 3**). This begs the question if oleosin is responsible for targeting LBs to the PM and PD. Oleosin is a structural LB protein that regulates LB size and stability, but which has enzymatic activity and may serve targeting functions. In *Arabidopsis* root hairs, which are devoid of PD except at their base, the transgenic overexpression of oleosin induces LBs that often remain circling in the cytoplasm, but also target the PM. In contrast, in leaf cells they are mostly found at PD, co-localizing with callose (**Figure 3**). Taken together, this suggests that PD are one of the end-destinations of LBs. Oleosin possesses a hydrophobic hairpin that anchors it to the LB core (Huang, 1996; Li et al., 2002). Although oleosin overexpression can induce so-called oleosin-bodies that are unrelated to LBs, it can promote LB formation from the ER in yeast (Jacquier et al., 2013) as well as in *Arabidopsis* root hairs (**Figure 3**). Thus, hairpin-containing plant proteins such as oleosin, and possibly the related LB protein caleosin, can induce LBs in a heterologous system. In line with this, the LB protein steroleosin, which does not have this capacity to induce LBs, is retained in the ER when expressed in protoplasts (De Domenico et al., 2011).

In an alternative route, macromolecular complexes might arrive at the PD channel via strands of ER that terminate at the desmotubule of the PD (Epel, 2009), or via the actin cytoskeleton (Oparka, 2004). Several viruses are known to highjack these systems to reach PD. The desmotubule, centrally located in the PD channel, could also be a potential target of LBs. For example, the protein reticulon which can induce extreme curvature in tubular cortical ER (Tolley et al., 2008) and which could possibly be present at the desmotubule (Tilsner et al., 2011), can associate with LBs (Krahmer et al., 2013). Thus, it seems possible that LBs deliver reticulon from their site of synthesis to the cortical tubular ER, as well as to the interconnected desmotubule.

PERSPECTIVE

Originally regarded as simple depots for neutral lipids, recent research has revealed that LBs are dynamic organelles that act as transport vehicles, signaling devices, and moving platforms for opportunistic travelers to various destinations, probably including PD. Elucidating LB–PD interactions might facilitate the identification of novel PD components as well as increase understanding of how these components are delivered to the interior of the PD. It could also facilitate the discrimination between structural and modulatory PD components and accidental visitors that are passing through the channel. In the near future, LB isolation, protein purification and sequencing is expected to generate inventories of putative LB-associated proteins. The validity of such inventories will require functional studies to confirm the putative role of LB-associated proteins in the regulation of the PD channel. It is anticipated that such endeavors will reveal that LBs contribute to the functional refurbishment of the PD chamber.

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Plasmodesmata without callose and calreticulin in higher plants – open channels for fast symplastic transport?

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Plasmodesmata (PD) represent membrane-lined channels that link adjacent plant cells across the cell wall. PD of higher plants contain a central tube of endoplasmic reticulum (ER) called desmotubule. Membrane and lumen proteins seem to be able to move through the desmotubule, but most transport processes through PD occur through the cytoplasmic annulus (Brunkard et al., 2013). Calreticulin (CRT), a highly conserved Ca^{2+}-binding protein found in all multicellular eukaryotes, predominantly located in the ER, was shown to localize to PD, though not all PD accumulate CRT. In nitrogen-fixing actinorhizal root nodules of the Australian tree *Casuarina glauca*, the primary walls of infected cells containing the microsymbiont become lignified upon infection. TEM analysis of these nodules showed that during the differentiation of infected cells, PD connecting infected cells, and connecting infected and adjacent uninfected cells, were reduced in number as well as diameter (Schubert et al., 2013). In contrast with PD connecting young infected cells, and most PD connecting mature infected and adjacent uninfected cells, PD connecting mature infected cells did not accumulate CRT. Furthermore, as shown here, these PD were not associated with callose, and based on their diameter, they probably had lost their desmotubules. We speculate that either this is a slow path to PD degradation, or that the loss of callose accumulation and presumably also desmotubules leads to the PD becoming open channels and improves metabolite exchange between cells.

Keywords: cell-to-cell communication, plasmodesmata, desmotubule, calreticulin, callose, callose synthase, pollen

PLASMODESMATA OF HIGHER PLANTS CONTAIN DESMOTUBULES AND ARE USUALLY ASSOCIATED WITH CALRETICULIN

Plasmodesmata (PD) represent membrane-lined channels that link adjacent plant cells across the cell wall and provide symplasmic connectivity, allowing the transfer of metabolites, RNAs, proteins, viruses, and even plastids (Thyssen et al., 2012). PD of higher plants contain a central tube of endoplasmic reticulum (ER) called the central rod or desmotubule. The surfaces of the desmotubule and of the plasma membrane are covered with globular particles interlinked with spokes, thereby stabilizing the internal structure of the PD and also limiting their lumen. Cell-to-cell movement of ER membrane dyes and even – proteins seems to be possible through the desmotubule (Martens et al., 2006; Guenoune-Gelbart et al., 2008), and in spite of its appressed form, molecules of up to 10.4 kDa can move through the ER lumen between neighboring cells in some cases (Barton et al., 2011). However, most transport processes through PD occur through the cytoplasmic annulus, the region between plasma membrane and desmotubule. Early studies suggested a size exclusion limit (SEL) of PD in the order of 1 kDa (Robards and Lucas, 1990), but this can be increased to up to 67 kDa in response to changes in the cytosolic Ca^{2+} concentration or interaction with specific proteins (Oparka et al., 1999; Stadler et al., 2005; Lucas, 2006). PD structure is highly dynamic; e.g., PD morphology can change from simple to branched during the sink source transition in leaves, concomitant with a decrease in SEL (Oparka et al., 1999; Roberts et al., 2001).

PD are assumed to have evolved in multicellular algae several times independently, including in Characeae, the ancestors of higher plants (Raven, 2005). Interestingly, not all multicellular algae have PD (Raven, 2005). The structure of algal PD differs from that of higher plants, most dramatically by the absence of a desmotubule in algal PD (Cook et al., 1997; Cook and Graham, 1999). However, there are reports on desmotubules in PD of Chlorophyceae and Characeae (*Uronema*, *Stigeoclonium*, *Chara*; Marchant, 1976; Brecknock et al., 2011).

Calreticulin (CRT), a highly conserved Ca^{2+}-binding protein found in all multicellular eukaryotes examined so far, is predominantly located in the ER (Michalak et al., 1999). CRT was also found in the Golgi (Borisjuk et al., 1998), and in animals also in the cytoplasm of certain cells (Dedhar, 1994), and at the cell surface (Johnson et al., 2001). CRT was shown to localize to PD in maize root apices (Baluška et al., 1999), as well as to PD in suspension cell cultures of tobacco and *Arabidopsis* (Laporte et al., 2003; Bayer et al., 2004), suggesting a role in cell-to-cell transport. This suggestion was supported by the finding that CRT interacts with a viral movement protein (Chen et al., 2005). However, root cap PD do not accumulate CRT (Baluška et al., 1999, 2000). Postmitotic cells of the root epidermis, which like root cap cells are symplasmically isolated, also do not accumulate CRT (Baluška et al., 1999, 2000, 2001), leading to the suggestion that

CRT might represent a marker for sink strength. However, CRT is also formed in response to different stresses, and detailed observations led to hypothesis that it represents a universal mediator of fast plasmodesmal closure (Bilska and Sowiński, 2010). It is not quite clear whether CRT is localized in the ER – i.e., near the beginning of the desmotubule – or in the cell wall (Baluška et al., 1999; Bayer et al., 2004); yet, a comparison of the immunolocalization of CRT and callose favors a localization in the ER (Bayer et al., 2004).

CALLOSE PLAYS A ROLE IN REGULATING THE SEL OF PD

The transport through PD can be regulated by the deposition of callose, a β-1,3-glucan, between the plasma membrane and the wall in the neck region where the cytoplasmic annulus is constricted (Bucher et al., 2001; Simpson et al., 2009). Callose production is catalyzed by callose synthases in the cell wall and is induced by biotic as well as abiotic stresses (Scheible and Pauly, 2004; Benitez-Alfonso and Jackson, 2009). The identification of mutants affected in cell redox homeostasis as well as in intercellular transport, and the observation of changes in symplastic permeability of tissues in response to treatment with oxidants, have been interpreted to suggest that intercellular transport is regulated in response to the production of reactive oxygen species (ROS) *via* callose formation (Benitez-Alfonso and Jackson, 2009; Benitez-Alfonso et al., 2011).

INFECTED CELLS IN ROOT NODULES OF *C. glauca* REDUCE THEIR PD CONNECTIONS TO ADJACENT CELLS IN THE COURSE OF DEVELOPMENT, WHICH IS ASSOCIATED WITH THE LOSS OF CRT ASSOCIATION

Nitrogen-fixing root nodules, specifically their infected cells that harbor the nitrogen-fixing bacterial microsymbionts which rely on the plant for carbon supply, represent carbon sinks and nitrogen sources. Analysis of the mechanisms of phloem photosynthate partitioning in actinorhizal nodules of the Australian tree *C. glauca* revealed that here, plasmodesmal connections between infected cells, and to a lesser degree between infected and uninfected cells, were reduced during the differentiation of infected cells (Schubert et al., 2013). This concerned the number as well as the diameter of PD. The numbers of PD connecting infected cortical cells were reduced more strongly than the numbers connecting infected to adjacent uninfected cortical cells (by 84 vs. 60%, respectively) but the reduction in diameter was similar in both cases (by 55 vs. 49%, respectively). Furthermore, PD connecting mature infected cortical cells did not accumulate CRT (Schubert et al., 2013; **Figures 1A,B**). CRT labeling was only in rare cases observed for PD connecting infected and adjacent uninfected cells, but was common for PD connecting uninfected cells (Schubert et al., 2013; **Figures 1C,D**).

Under the assumption that CRT is localized in the ER at the opening of the PD, its absence might imply the absence of desmotubules. Desmotubule membranes are the closest juxtaposed lipid bilayers known in nature, 10–15 nm in diameter at their most constricted (Burch-Smith and Zambryski, 2012). Thus, in PD with a diameter of 22 or 26 nm, respectively, the absence of desmotubules should not be surprising, particularly in view of the fact that proteinaceous spokes should protrude from the desmotubule, and globular particles from the plasma membrane in the cytoplasmic sleeve (Burch-Smith and Zambryski, 2012). Loss of desmotubules has also been observed in nematode-parasitized root cortical cells from clover (*Trifolium incarnatum*) and tomato (*Solanum esculentum*), but here this phenomenon was associated with an increase in PD diameter (Hussey et al., 1992).

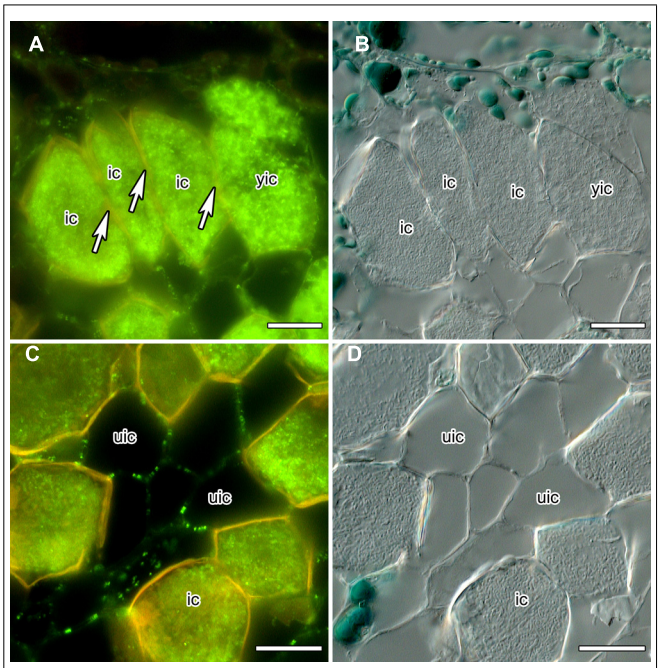

FIGURE 1 | Immunolocalization of calreticulin (CRT) in longitudinal sections of *C. glauca* nodules embedded in Steedman's wax (for the method, see Zdyb et al., 2011; for the antibody, see Baluška et al., 1999). Fluorescence micrographs **(A,C)** and differential interference contrast micrographs **(B,D)** are shown. The lignified walls of infected cells fluoresce in yellow under blue light. **(A,B)** No CRT labeling is found in walls (see arrow) between infected cells (ic). **(C,D)** CRT is labeled in walls connecting uninfected cells (uic) in a punctate pattern. Size bars denote 20 μm.

PD OF MATURE INFECTED CELLS OF *C. glauca* NODULES DO NOT SHOW CALLOSE ACCUMULATION

In order to obtain more information on the special features of the PD between infected cells, we analyzed the distribution of callose and of callose synthase. The gradual decrease of PD diameter during the differentiation of infected cortical cells of *C. glauca* nodules was associated with the loss of callose accumulation at PD connecting infected cells, or infected and adjacent uninfected cells, as detected by Aniline blue staining (**Figure 2A**). Aniline blue staining of callose was common for PD connecting uninfected cortical cells (**Figure 2B**). In an attempt to confirm the absence of callose at PD connecting infected cells, an antibody raised against callose synthase from *Nicotiana alata* pollen tubes (Cai et al., 2011) was used. The antibody labeled small granules in the plasma membranes of the youngest cells of the nodule lobe, close to the meristem (**Figure 2C**). Punctate labeling adjacent to the cell walls between uninfected cortical cells in the area of mature infected cells was also found (**Figure 2D**); however, no labeling was observed in walls of

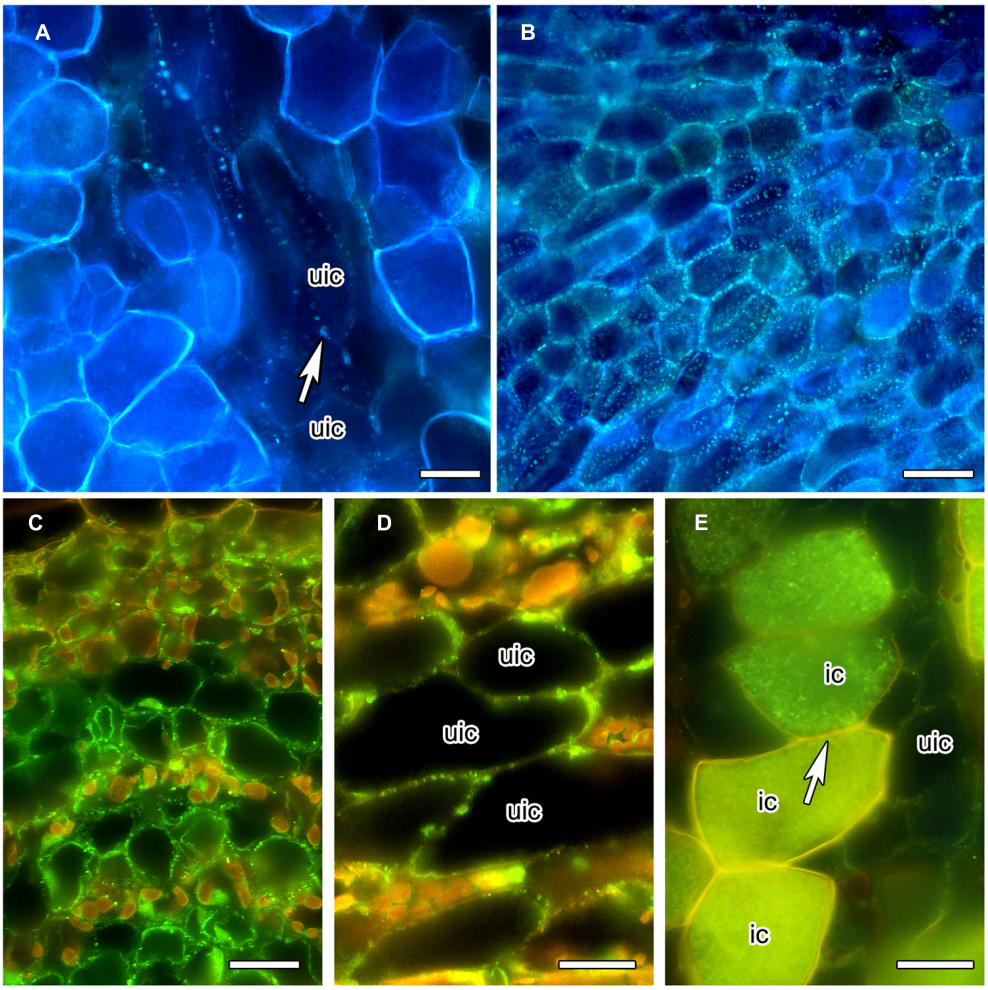

FIGURE 2 | Anilin Blue staining of callose (A,B) in longitudinal vibratome sections of living *C. glauca* nodules embedded in agarose (Brundrett et al., 1988), and immunolocalization of callose synthase (C–E) in cross sections of fixed nodules embedded in Steedman's wax (for the method, see Zdyb et al., 2011; for the antibody, see Cai et al., 2011). Micrographs were taken under fluorescent light. The lignified walls of infected cells show in white under UV light **(A,B)** and in yellow under blue light **(C–E)**. **(A,B)** Punctate Anilin Blue-staining of callose (white fluorescence) is found in the walls connecting uninfected cells (uic; white arrow), but no labeling is found in infected cells [recognizable by their fluorescent cell walls; labeling should be visible because the fluorescence of Anilin Blue, as obvious in **(B)** which shows a younger area of the cortex before the onset of infection, is more yellowish then the fluorescence of the walls of infected cells]. **(C)** Callose synthase is detected in the plasma membranes of uninfected cortical cells in the youngest part of the cortex, close to the apical meristem. In the older part of the cortex – **(D)** shows the outer cortex in the zone of nitrogen fixation – the labeling is less dense. **(E)** No labeling was seen in the plasma membranes of infected cells (ic; arrow). Size bars denote 20 μm in **(A–D)** and 25 μm in **(E)**.

infected cells (**Figure 2E**). Since callose synthases are encoded by a gene family (Verma and Hong, 2001), no firm conclusions can be drawn regarding the potential presence of callose at PD connecting infected cells of *C. glauca* nodules; yet, the localization of callose and the immunolocalizations of callose synthase are consistent.

MATURE INFECTED CELLS OF *C. glauca* ARE APOPLASTICALLY ISOLATED

Interestingly, *C. glauca* infected cells should depend on symplasmic supply with photosynthates since apoplastic transport pathways a blocked by the impregnation of the walls of infected cells with a very hydrophobic type of lignin (Berg and McDowell, 1988; Schubert et al., 2013). It has been suggested that the properties of the cell wall surrounding PD may restrict the degree to which the microchannels can dilate (Kragler et al., 1998). Therefore, this lignification, which commences upon infection by the microsymbiont, is the most likely explanation for the observed reduction of PD diameter in *C. glauca* infected cells. While many cases are known where lignification or suberization of cell walls does not affect the PD traversing these cell walls, in such cases the PD are organized in primary pit fields, i.e., areas with reduced thickness of the primary wall and without secondary cell wall deposition (Robinson-Beers and Evert, 1991) and with, it seems, a distinctive cell wall composition (Orfila and Knox, 2000), while in infected cells of *C. glauca* nodules, the primary walls are lignified, i.e., the PD traverse the lignified parts of the wall.

WHAT ARE THE EFFECTS OF THE CHANGES IN PD OF INFECTED CELLS IN *C. glauca* NODULES ON SYMPLASTIC TRANSPORT?

The diameters of PD connecting infected cells with adjacent infected or uninfected cells are significantly reduced compared to those connecting uninfected cells (Schubert et al., 2013). The lack of callose and callose synthase would mean the absence of negative regulation of the SEL of these PD by callose deposition. Similarly, no callose deposition was observed along cell walls between giant cells in nematode feeding sites of tobacco, but callose deposition was found frequently along cell walls toward neighboring cells (Hofmann et al., 2010).

The complete lack of CRT labeling at PD connecting infected cells, and the rarity of CRT labeling at PD connecting infected and adjacent uninfected cells, cannot be linked to the hypothesis that CRT represents a marker for sink strength (Baluška et al., 2001) since these cells, which express sucrose synthase at high levels, are strong sinks (Schubert et al., 2013). Furthermore, the infected cells are microaerobic (Berg and McDowell, 1987; Schubert et al., 2013) and usually, ATP depletion leads to the opening of PD (Cleland et al., 1994). However, the loss of CRT labeling is consistent with the hypothesis that CRT is a mediator of fast plasmodesmal closure. First, the PD closure mechanism involving CRT might not be able to function in PD traversing lignified primary cell walls. Second, since the infected cells are apoplastically isolated, additional symplastic isolation would mean cell death, and therefore should not be too easy to install.

It seems likely the changes in PD traversing the walls of infected cells are linked to the lignin deposition in the primary walls of infected cells. This can be interpreted in two ways. Either, the effect of lignification might be the gradual loss of function of PD, adding symplastic isolation to the apoplastic isolation of infected cells of *C. glauca* nodules. In that case, infection would eventually lead to cell death. Alternatively, the shrinking of the PD diameter could be compensated for by the loss of the desmotubules that might be implied by the loss of CRT labeling. Thanks to the central desmotubule, globular particles on the plasma membrane that lines the channel, and spoke-like connections between the desmotubule and the plasma membrane, the operational diameter of PD is no larger than 2 (Van Bel, 1993) or 3 nm (Kragler, 2013). Thus, the disappearance of the desmotubule could be expected to correlate with the disappearance of the spokes, increasing the operational diameter to one that is higher than of normal PD. Hence, the lack of desmotubules and callose would transform the PD to wide open channels for symplasmic transport. That would be consistent with the only known example for loss of PD desmotubules in plant cells, namely in nematode-parasitized root cortical cells from clover (*T. incarnatum*) and tomato (*S. esculentum*; Hussey et al., 1992).

PD OF INFECTED CELLS OF *C. glauca* NODULES – ON THE WAY TO COMPLETE CLOSURE, OR OPEN CHANNELS FOR OPTIMIZED SYMPLASTIC TRANSPORT?

In order to test which of these hypotheses is correct, a construct expressing green fluorescent protein (GFP) under control of a promoter specific to uninfected nodule cortical cells could be used. So far, no promoter driving expression specific to uninfected root cortical cells of *C. glauca* nodules has been characterized; however, Perrine-Walker et al. (2011) described an auxin efflux carrier (CgPIN1) that was present specifically in uninfected, not in infected nodule cortical cells. Hence, the promoter of *CgPIN1* could be used to drive the expression of *GFP* in uninfected nodule cortical cells. With a molecular mass of 27 kDa and a Stokes radius of 1.8 nm, GFP can travel through the PD connecting root cortical cells (Stadler et al., 2005) and should also be able to pass through the PD connecting infected with adjacent uninfected cortical cells of *C. glauca*. A β-glucuronidase (GUS) fusion construct with the same promoter could serve as negative control, since with a molecular mass of 68 kDa and a Stokes radius of 3.3 nm (Fisher and Cash-Clark, 2000), GUS cannot travel through most PD (Fukuda et al., 2005). If cytological analysis shows the presence of GFP in infected cells of transgenic nodules, larger GFP constructs could be tested for a precise assessment of the SEL of the PD of infected cells.

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Homotypic fusion of endoplasmic reticulum membranes in plant cells

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The endoplasmic reticulum (ER) is a membrane-bounded organelle whose membrane comprises a network of tubules and sheets. The formation of these characteristic shapes and maintenance of their continuity through homotypic membrane fusion appears to be critical for the proper functioning of the ER. The atlastins (ATLs), a family of ER-localized dynamin-like GTPases, have been identified as fusogens of the ER membranes in metazoans. Mutations of the ATL proteins in mammalian cells cause morphological defects in the ER, and purified Drosophila ATL mediates membrane fusion *in vitro*. Plant cells do not possess ATL, but a family of similar GTPases, named root hair defective 3 (RHD3), are likely the functional orthologs of ATLs. In this review, we summarize recent advances in our understanding of how RHD3 proteins play a role in homotypic ER fusion. We also discuss the possible physiological significance of forming a tubular ER network in plant cells.

Keywords: endoplasmic reticulum, membrane proteins, membrane fusion, GTPase, plant development

INTRODUCTION

The endoplasmic reticulum (ER) is the origin of the endomembrane system in eukaryotic cells. Secretory proteins and most of the integral membrane proteins are synthesized and folded by the ER, cellular membrane sources are generated on the ER, and calcium ions are stored in the lumen of the ER. Morphologically, the ER membranes can adopt a tubular shape or form flattened cisternal structures, called ER sheets (Shibata et al., 2006). Despite the ER representing one of the largest intracellular membrane surfaces, the ER membranes in each cell are continuous as one entity. Though the sheets may be stacked by helicoidal membrane motifs (Terasaki et al., 2013), tubules often extend from sheets and are connected via three-way junctions into a reticular network (Lee and Chen, 1988; Hu et al., 2008). In some areas of the ER, tubules and sheets are interspersed in fenestrated structures (West et al., 2011).

The formation and maintenance of a continuous membrane system requires constant fusion of identical membranes. A similar fusion process includes the merger of mitochondrial membranes, in which dynamin-like GTPases mitofusin/Fzo1 and OPA1/Mgm1 play important roles (Chan, 2006; Hoppins et al., 2007). However, how such homotypic fusion occurs is poorly understood. In contrast, the merger of heterotypic membranes, such as the fusion of viral and cellular membranes or transport vesicles with target membranes, has been studied intensively. In viral fusion, the membranes are pulled together by an intramolecular conformational change in a single protein (Harrison, 2008). In vesicular fusion, three t-SNARE proteins in one membrane and a v-SNARE partner in the other zipper up to form a four-helix bundle in the fused lipid bilayer (Jahn and Scheller, 2006; Martens and McMahon, 2008; Wickner and Schekman, 2008; Sudhof and Rothman, 2009).

In mammalian cells, defects in branch formation of the ER network, a sign of a lack of sufficient homotypic fusion, was recently linked to a class of membrane-bound, dynamin-like GTPases named atlastins (ATLs; Rismanchi et al., 2008; Hu et al., 2009). Lipid bilayer fusion can be achieved with purified Drosophila ATL (Orso et al., 2009; Bian et al., 2011). Following the discovery of ATLs, Sey1p in yeast cells was identified as a functional ortholog (Anwar et al., 2012). The deletion of Sey1p drastically delays ER fusion during mating, and the re-introduction of Sey1p restores the defects (Anwar et al., 2012). Similar to ATL, reconstituted Sey1p is capable of fusing vesicles *in vitro* (Anwar et al., 2012).

Plant cells do not possess ATL homologs; however, a GTPase called root hair defective 3 (RHD3) is related to Sey1p in regards to sequence (Brands and Ho, 2002) and has been suggested to mediate the fusion of ER membranes (Hu et al., 2009; Chen et al., 2011). Although the mechanisms of ER fusion may be conserved in plant cells, the plant ER exhibits several unique features: a prominent cortical ER (West et al., 2011); participation in plasmodesma formation, a specialized intercellular ER connection (Gupton et al., 2006); and the movement of Golgi bodies along ER tubules (Boevink et al., 1998; Faso et al., 2009; Sparkes et al., 2009). These characteristics imply that homotypic ER fusion in plant cells may play distinct roles.

HOMOTYPIC ER FUSION IN MAMMALIAN AND YEAST CELLS

The first clue of homotypic fusion of the ER membranes in mammalian cells came from overexpression of mutant forms of ATL, a membrane-bound GTPase (Rismanchi et al., 2008). ATL mutations cause unbranched ER morphology, indicating a lack of fusion between ER tubules. ATL is anchored in the membrane

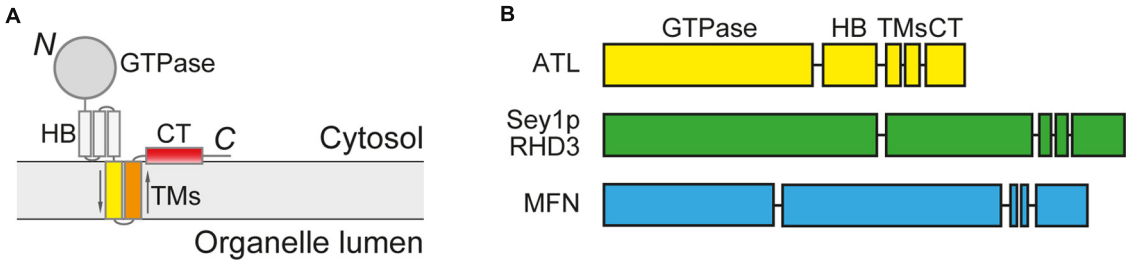

FIGURE 1 | Membrane topology and domain structure of ATL-related GTPases. (A) Membrane topology of ATL-related GTPases. 3HB, three-helix bundle; TMs, transmembrane segments; CT, C-terminal tail. **(B)** Domain structure of ATL-related GTPases.

by two closely spaced transmembrane (TM) segments, exposing both the N-terminal GTPase domain and the C-terminal tail (CT) to the cytosol (**Figure 1A**). ATL localizes mostly in the tubular region of the ER and interacts with the ER tubule resident proteins reticulons and DP1/Yop1p (Hu et al., 2009), two families of integral membranes that induce high curvature in the ER membranes to form tubules (Voeltz et al., 2006; Hu et al., 2009). ATL belongs to the dynamin superfamily of GTPases. A related family member, mitofusin (MFN), shares membrane topology and domain structures with ATL (**Figure 1B**) and is known to mediate fusion of the outer mitochondrial membranes (Hermann et al., 1998; Chen et al., 2003). Thus, ATL is likely responsible for fusion of the ER membranes. In fact, depletion of ATL causes unbranched ER in mammalian cells (Hu et al., 2009), and ER fragmentation in *Drosophila* (Orso et al., 2009). In addition, antibodies against ATL block ER network formation *in vitro* in Xenopus egg membrane extracts (Hu et al., 2009). Most convincingly, purified *Drosophila* ATL is able to mediate vesicle fusion *in vitro* when reconstituted into proteoliposomes (Orso et al., 2009).

Atlastins is conserved in most metazoa, but it is not found in many organisms in which the tubular ER network is properly formed. *Saccharomyces cerevisiae* is among these organisms, but a similar GTPase named Sey1p was recently identified as an ER fusogen (Anwar et al., 2012). Sey1p is a synthetic enhancer for Yop1p (Brands and Ho, 2002), one of the ER tubule shaping proteins, and it not so surprisingly plays a role in tubular ER network formation. Cells lacking Sey1p exhibit minor morphological defects in the ER (Hu et al., 2009), but an ER SNARE Ufe1p is required when SEY1 is deleted and may represent an alternative ER fusogen in yeast (Anwar et al., 2012).

ASSAYS FOR HOMOTYPIC ER FUSION

Several assays have been developed or adapted to verify candidates for homotypic ER fusion. The first assay visualizes the integrity of the ER in yeast cells (Hu et al., 2009). Deletion of Sey1p and either Rtn1p or Yop1p, membrane proteins that shape the ER tubules, causes drastic ER defects; in particular, the tubular ER network is mostly converted to sheets and the large areas of the cortex are void of ER, indicating a lack of ER fusion. When wild-type Sey1p is re-introduced, fusion activities in the ER resume and ER morphology, visualized by GFP-labeled ER-resident protein Sec63p, restored. The rescue of the ER defects in *sey1*Δ *rtn1*Δ or *sey1*Δ *yop1*Δ cells by certain proteins indirectly indicates its ability to mediate ER fusion.

The second assay monitors ER fusion during the mating of yeast cells (Anwar et al., 2012). Similar assays have been used to study nuclear fusion or mitochondrial fusion. Haploid yeast cells expressing cytosolic GFP remated with cells expressing a red fluorescent protein (RFP)-containing ER marker (ss-RFP-HDEL). When cell fusion occurs between two types of cells, the cytosolic GFP of one cell rapidly diffuses to the other cell, marking the starting point for ER fusion. The efficiency of ER fusion is monitored by the equilibration of the RFP signal between two cells. Cells lacking Sey1p exhibit a significant delay in ER fusion, but plasmid-driven expression of Sey1p would restore such a defect. To test whether certain molecules mediate ER fusion *in vivo*, they are expressed in haploid cells with either cytosolic or ER marker, both of which lack Sey1p. ER fusion is then measured and compared to that of untransformed *sey1*Δ cells.

Finally, *in vitro* fusion assays have been adapted from studies of SNARE-mediated fusion (Weber et al., 1998; Scott et al., 2003). For lipid mixing tests, full-length fusion candidates are purified in detergent and reconstituted into proteoliposomes upon detergent removal. A group of vesicles incorporates lipids labeled with two fluorophores (NBD and rhodamine) at quenching concentrations. When these vesicles fuse with vesicles containing unlabeled lipids, the labeled lipids are diluted and subsequently dequenched. The increase in fluorescence correlates with the level of lipid mixing resulting from fusion. To further distinguish hemi-fusion and full fusion, two fluorescent dyes are incorporated as a FRET pair into reconstituted vesicles and the FRET signal measured as an indicator of the content mixing resulting from full fusion of the two bilayers (Zucchi and Zick, 2011).

Combining these three assays, ATL and Sey1p proteins were tested and confirmed to mediate fusion of the ER membranes (**Table 1**). Based on the results for *Drosophila* ATL, the fusion reaction can lead to efficient content mixing with nearly no lysis of the membranes (Liu et al., 2012). Using the same criteria, RHD3 has recently joined the list of ER fusogens as a plant ortholog of ATL and Sey1p (Zhang et al., 2013).

RHD3 FAMILY PROTEINS AS PLANT ER FUSOGENS

The components involved in shaping ER tubules are conserved among eukaryotes. Shortly after reticulons and DP1/Yop1p

Table 1 | Assays for homotypic ER fusion.

GTPases	ER morphology in yeast cells	In cell fusion in yeast cells	Lipid mixing *in vitro*	Content mixing *in vitro*
ATL	✓Anwar et al. (2012)	✓Anwar et al. (2012)	✓Orso et al. (2009)	✓Liu et al. (2012)
Sey1p	✓Hu et al. (2009)	✓Anwar et al. (2012)	✓Anwar et al. (2012)	ND
RHD3	×Chen et al. (2011), ✓Zhang et al. (2013)	✓Zhang et al. (2013)	✓Zhang et al. (2013)	ND

✓, *Functional;* ×, *Non-functional; ND, not determined.*

were found in mammals and yeast cells (Voeltz et al., 2006), the plant orthologs were analyzed and confirmed to have the same role (Nziengui et al., 2007; Sparkes et al., 2010). Similarly, when ATL and Sey1p were shown to mediate ER fusion (Hu et al., 2009), a related protein family, RHD3, became very plausible candidates for ER fusogens in plant cells.

RHD3 was initially discovered by a genetic screen of root hair development (Schiefelbein and Somerville, 1990). Mutations in RHD3 proteins cause short and wavy root hairs and a dwarf phenotype (Schiefelbein and Somerville, 1990; Wang et al., 1997). A role for RHD3 in ER morphogenesis was indicated, even before the characterization of ATL and Sey1p, when *rhd3-1* plants (A575V) were found to contain "cable-like" ER (Zheng et al., 2004), a defect reminiscent of ATL mutations or depletion in mammalian cells. Subsequently, several other RHD3 point mutants or null mutants were found to result in the same ER defects (Zheng et al., 2004; Chen et al., 2011; Stefano et al., 2012; Zhang et al., 2013), supporting the notion that RHD3 plays a role in connecting ER tubules. In addition to RHD3, two RHD3-like proteins were found in *Arabidopsis* (Hu et al., 2003). RL1 is expressed only in pollen, whereas RL2 is expressed ubiquitously, but both are present at very low levels. Individual deletions of the RL proteins show no detectable defects in plant development. However, over-expression of RL2 rescues the *rhd3-1* mutant (Chen et al., 2011), suggesting a redundant role among these proteins.

Similar to ATL and Sey1p, RHD3 localizes mainly to the tubular ER network; colocalizes with HVA22 (Chen et al., 2011), a plant ortholog of DP1/Yop1p; and its homolog RL2 interacts with plant reticulons (Lee et al., 2013). However, RHD3 and Sey1p are not thought to be interchangeable (Chen et al., 2011), i.e. Sey1p cannot rescue the rhd3 mutant, and RHD3 cannot replace Sey1p in yeast. To test the possibility that RHD3 and Sey1p act differently, the yeast complementation assay was recently revisited. Either RHD3 or the RL proteins was expressed under the control of the endogenous SEY1 promoter in *sey1*Δ *yop1*Δ cells, and the results indicated that RHD3 family members are capable of restoring ER defects in *sey1*Δ *yop1*Δ cells (Zhang et al., 2013). Though Sey1p might not be functional in the setting of plant cells, these findings suggest that RHD3 and Sey1p act similarly in yeast cells and *in vitro* as purified proteins. Using the same assays that are applicable to ATL and Sey1p, the RHD3 proteins fuse the ER in cells and lipid membranes *in vitro*, confirming that they are ER fusogens in plant cells(Zhang et al., 2013).

Some fusion events have been observed between peripheral ER tubules in plant cells lacking RHD3 (Stefano et al., 2012). As neither RL is dispensable on the rhd3-null background (Stefano et al., 2012), these fusion activities are likely carried out by RL proteins, even though their levels are very low compared to RHD3. The strong ER-branching defect in *rhd3* mutants suggests that RHD3 is the major force to connect ER membranes, but whether another ER fusogen exists in plant cells remains to be tested.

MECHANISMS FOR HOMOTYPIC ER FUSION IN PLANT CELLS

Given the domain structure and functional similarity among ATL, Sey1p, and RHD3, these GTPases likely utilize conserved mechanisms to mediate fusion. How RHD3 performs the fusion reaction in plant cells is not clear, but significant progress has been made in understanding ATL-mediated homotypic fusion. Crystal structures of the N-terminal cytosolic domain of human ATL1 have been determined (Bian et al., 2011; Byrnes and Sondermann, 2011), revealing a GTPase domain and three-helix bundle (3HB) connected by a linker region. The GTPase domains face each other to form a nucleotide-dependent dimer in all structures, but the 3HBs are positioned differently. In the structure obtained when only GDP is added, the 3HBs following the GTPase domains point in opposite directions; in another structure in which GDP and phosphate are present, the 3HBs are parallel to one another and crossover to dock against the GTPase domain of the partner molecule; and in the recent structures in which GMPPNP, a non-hydrolysable analog of GTP, or GDP and aluminum fluoride were used, the 3HBs come even closer in the crossed over conformation (Byrnes et al., 2013). Taken together, the evidence indicates that GTP binding induces interactions between ATL molecules across the apposing membranes, and subsequent GTP hydrolysis causes conformational changes through steps that are not entirely clear to force the 3HBs of engaging ATL molecules come very close, pulling the two membranes together.

In addition to the GTP-dependent mechanism, the CT of ATL forms an amphipathic helix that binds and perturbs the membrane bilayer, facilitating the fusion process, and the TM segments are required for efficient fusion, probably by mediating nucleotide-independent oligomerization of ATL molecules (Liu et al., 2012).

The homotypic interactions of RHD3 protein have been confirmed (Chen et al., 2011), but the mechanisms of RHD3-mediated membrane fusion remain to be tested. Notably, the region between the GTPase domain of RHD3 and the TM segments is much longer than that of the 3HB in ATL. Based on secondary structure prediction, this region likely forms a helical bundle, but whether it binds to the GTPase domain, or even in a similar manner as ATL, is largely unknown.

PERSPECTIVE

Homotypic ER fusion appears to be a conserved process among eukaryotic cells. In plants, the process is mediated primarily by the RHD3 family of proteins. Like the ATL family of proteins, members of the RHD3 family are present ubiquitously. However, the prominent defects caused by mutations in RHD3 occur in cells with long protrusions, namely the root hairs, which is reminiscent of ATL1 with cortical spinal motor neurons. Though complete deletion of ATLs in mammals is yet to be achieved, the loss of RHD3 and either of the RL proteins results in lethality (Zhang et al., 2013). These results suggest that, at least in plants, more cell types require the presence of the RHD3 family than is previously thought.

One important question that remains to be addressed is the role of the RHD3 family *in vivo*. For example, there may be a link between these GTPases and the ER-plasma membrane (PM) contact sites. The non-branched or "cable-like" ER is often clustered in the cell body rather than present in the cortex. It is reasonable to speculate that RHD3 mutations perturb the general functions of the ER-PM contact sites, such as calcium signaling or lipid sensing and transfer. The formation of plasmodesmata also relies on coordination of the PM and cortical ER, and ER tubules from neighboring cells need to be fused in the nanopores. Will plasmodesmata be properly generated in RHD3 mutants? If not, what happens to the nanopores? Finally, Golgi body distribution and movement along the tubular ER network has been shown to be affected in rhd3-1 (Chen et al., 2011) or gom8 (Stefano et al., 2012), an EMS mutant of RHD3 (P701S). Does this defect directly lead to impaired plant development? The hope is that answering these questions will have an impact on our understanding of the correlation between the morphology of organelles and their functions.

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Evolutionary aspects of non-cell-autonomous regulation in vascular plants: structural background and models to study

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Plasmodesmata (PD) serve for the exchange of information in form of miRNA, proteins, and mRNA between adjacent cells in the course of plant development. This fundamental role of PD is well established in angiosperms but has not yet been traced back to the evolutionary ancient plant taxa where functional studies lag behind studies of PD structure and ontogenetic origin. There is convincing evidence that the ability to form secondary (*post-cytokinesis*) PD, which can connect any adjacent cells, contrary to primary PD which form during cytokinesis and link only cells of the same lineage, appeared in the evolution of higher plants at least twice: in seed plants and in some representatives of the Lycopodiophyta. The (in)ability to form secondary PD is manifested in the symplasmic organization of the shoot apical meristem (SAM) which in most taxa of seedless vascular plants differs dramatically from that in seed plants. Lycopodiophyta appear to be suitable models to analyze the transport of developmental regulators via PD in SAMs with symplasmic organization both different from, as well as analogous to, that in angiosperms, and to understand the evolutionary aspects of the role of this transport in the morphogenesis of vascular plant taxa.

Keywords: Lycopodiophyta, primary plasmodesmata, secondary plasmodesmata, cell boundaries, shoot apical meristem, KNOX transcription factors

THE APPEARANCE OF PD IN EVOLUTION

Plasmodesmata (PD) are tiny channels of several tenths to hundreds of nanometers in diameter which connect adjacent cells providing continuity of their cytoplasm and plasma membranes, and in most cases also continuity of their endoplasmic compartments and endoplasmic reticulum (ER) membranes due to the presence of the desmotubules. In multicellular organisms, PD provide a convenient route for cell-to-cell communication among immobile cells surrounded by rigid cell walls. In fact, PD occur in land plants, in multicellular algae and phototrophic protists (but astonishingly not in all groups; reviewed by Raven, 2005), as well as in a few species of multicellular fungi (reviewed by Lucas et al., 1993). An excellent review by Raven (2005) indicates that PD evolved independently in brown algae, in characean algae, and up to five times in green algae. Although truly multicellular forms can be found also among red algae, haptophytes and dinoflagellates, they do not form PD.

Depending on the stage of the cell cycle during which they appear, PD are classified as primary, or cytokinetic, and secondary, which form *post-cytokinesis*. Primary PD can be secondarily modified later by addition of new branches, or serve as templates for secondary PD formation (Ehlers and Kollmann, 2001; Faulkner et al., 2008). The mechanism of primary PD formation strictly correlates with the mechanism of cell division: primary PD occur between cells which divide via the formation of a cell plate and not via furrowing (Stewart et al., 1973), with the possible exception of a few brown algae (Katsaros et al., 2009). In characean algae and embryophytes, after separation of the nuclei, the microtubules of the mitotic spindle remain perpendicular to the plane of cell division where the nascent phragmoplast forms. Cisterns of the ER are laid along these microtubules and serve as templates for the primary PD, becoming the desmotubules later on. Interestingly, in the PD-forming fungus *Rhizopus sexualis* cell divisions occur similarly to those of land plants, i.e., via the formation of a phragmoplast, with the difference that the cell plate forms centripetally and not centrifugally; PD in this fungus contain desmotubules (Hawker and Gooday, 1967). In green algae, the cell plate formation is accompanied (after the mitotic spindle has disappeared) by the formation of a system of microtubules oriented parallel to the plane of cell division, called phycoplast. Although there is no obvious mechanism for insertion of desmotubules in PD, in a few green algae, e.g., *Ulothrix* and *Stigeoclonium* (Floyd et al., 1971) PD were shown to contain desmotubules. In brown algae, cell plate formation involves neither phycoplast nor phragmoplast; an elegant study on *Dictyota dichotoma* has shown that the pre-PD, probably synthesized in the cytoplasm as whole complex structures, are introduced into membranous sacs positioned at the place of the future cell plate (Terauchi et al., 2012). The PD in brown algae typically do not contain desmotubules (Katsaros et al., 2009).

The occurrence of secondary PD in the absence of primary PD has been reported for several algae. One example is *Chara corallina* where simple PD lacking desmotubules are formed as holes appearing in already existing cell walls (Franceschi et al., 1994). Interestingly, Brecknock et al. (2011) recently reported the presence of desmotubules in PD of the same species. In *Chara*

zeylanica, the formation of desmotubules-containing primary PD resembling those in embryophytes has been observed while no secondary PD have been found (Cook et al., 1997). Thus, there are characean algae with only primary PD, as well as characean algae with only secondary PD; to our knowledge, no characean algae capable of forming both primary and secondary PD, similarly to seed plants, have been found thus far.

An interesting hypothesis on the appearance of PD in plant evolution has been proposed by Niklas (2000). He argued that "some of the features characterizing the multicellular plant body, such as cellular differentiation, PD-like structures, control mechanisms for the orientation of cell cleavage, and cellular differentiation, are evident among extant and presumably very ancient cyanobacteria." Thus, such traits like cell wall and PD might have evolved as parts of the phenomenon of multicellularity which could have been transmitted from cyanobacterial endosymbionts to the host nucleus via lateral transfer of genes "during or shortly after primary endosymbiotic events in the very distant past" (Niklas, 2000). Other authors argued that this seems rather unlikely, as the intercellular connections in cyanobacteria are more similar to gap junctions than to PD (Raven, 2005). However, the newest findings indicating that the process of PD formation in plant embryos depends to some extent on plastid signals (Burch-Smith and Zambryski, 2010; Burch-Smith et al., 2011), support Niklas' hypothesis.

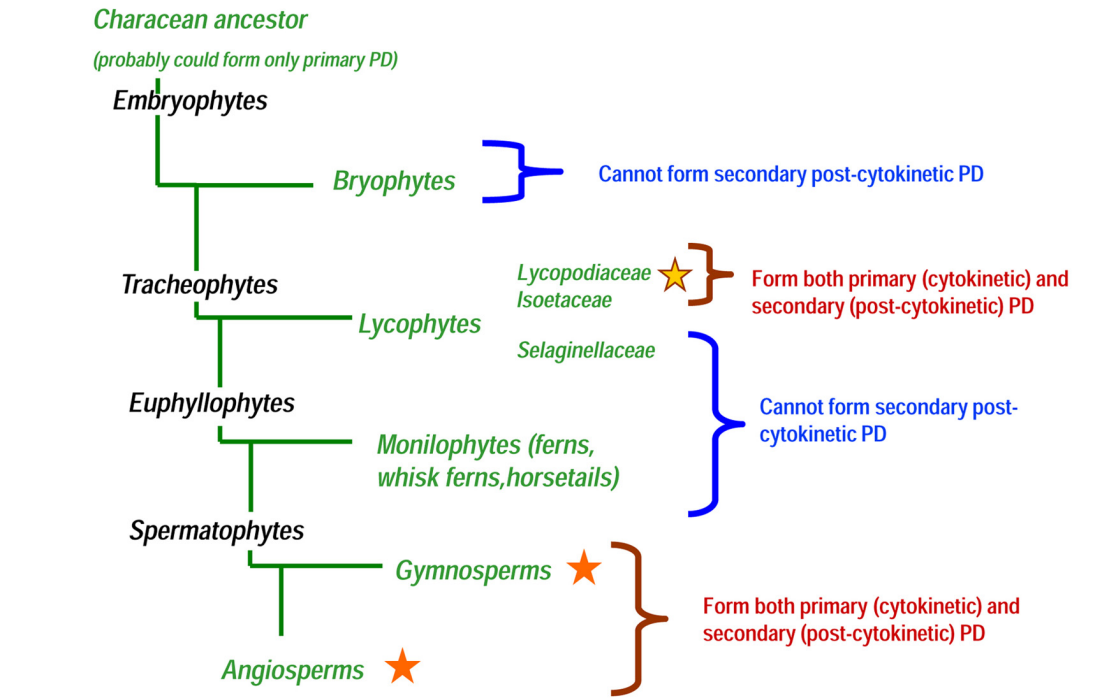
Several first class transmission electron microscopy (TEM) studies on PD in algae revealed a range of different internal structures which obviously reflect different phylogenetic and ontogenetic origin of these PD (Fraser and Gunning, 1969; Franceschi et al., 1994; Cook et al., 1997; Brecknock et al., 2011; Terauchi et al., 2012 and others). It can be expected that the composition of the cell wall, as well as the chemistry of the lipids in plasma membrane and in the ER for the desmotubule-containing PD, will have an impact on the mechanisms of PD functioning, e.g., regulation of the size exclusion limit (SEL), which might be very different in PD of different origin. So far, no data on the role of PD in the regulation of development are available for algae and fungi.

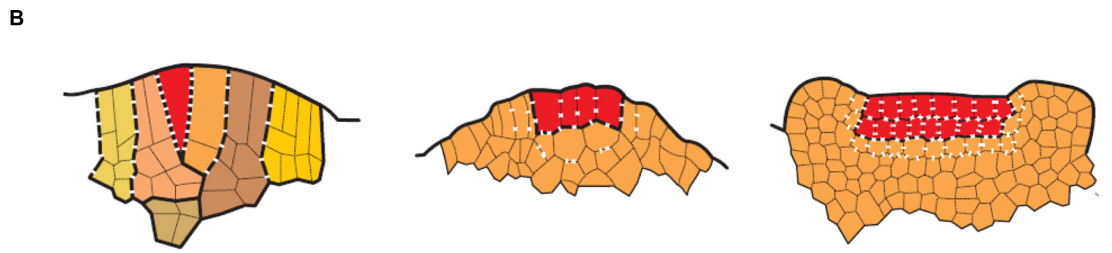
WHICH FACTORS MAY INFLUENCE PD FORMATION AND FUNCTIONS IN SEEDLESS VASCULAR PLANTS AS COMPARED TO SEED PLANTS?

Embryophytes have evolved from a characean ancestor (**Figure 1A**) but there are significant differences in membrane lipids and cell wall composition, as well as in the hormonal regulatory networks, between charophytes at the base and angiosperms at the top of land plant evolution, which can potentially impact the structure and function of PD in different taxa of embryophytes. PD of angiosperms are the best studied among land plants in terms of substructure, formation, and function. Their important properties include the presence of two closely juxtaposed membranes of different origin, the plasma membrane and the desmotubule, which probably contain special lipid domains, e.g., sphingolipid-enriched lipid rafts (Tilsner et al., 2011); the enclosure into a cell wall sheath which consists of the non-esterified pectin homogalacturonan and of callose and is devoid of cellulose and hemicelluloses (Heinlein and Epel, 2004); the ability to facilitate the intercellular spread of viral RNA, proteins and whole virions, as well as to transport non-cell-autonomous proteins and regulatory RNA, and the ability to reversibly change their diameter ("to gate"), possibly via regulation of callose synthesis and degradation (Lucas et al., 1993, 1995; Epel, 2009).

In seedless plants, the knowledge on cell wall composition, lipidomics, and hormonal regulatory networks is limited compared to angiosperms. However, genome sequencing in the moss *Physcomitrella patens* and the spike moss *Selaginella moellendorffii* has recently provided a solid basis for future analyses of evolution and diversity of these and other aspects in land plants. Several points might be potentially important for the study of PD function in non-angiosperms, as concluded from the data for angiosperms. One is the composition of the cell wall which sometimes influences the structure of PD, as found, e.g., in sugarcane where the presence of suberin lamellae resulted in the constriction of the desmotubules in PD (Robinson-Beers and Evert, 1991). Also, as mentioned above, in angiosperms PD were shown to be surrounded by a cell wall sheath of specific composition devoid of cellulose and enriched in low-esterified homogalacturonan: this was demonstrated by Roy et al. (1997) for PD in ripe apple and by Sutherland et al. (1999) for PD in kiwi fruit (reviewed in Heinlein and Epel, 2004). The reason for this is not known; however, it can be speculated that the presence of these specific cell wall constituents is important for PD biogenesis and/or function. No data on cell wall domains around PD are available for non-angiosperms thus far.

Cell wall composition in land plants shows significant variations. Characean algae possess only primary cell walls which do not contain lignin or rhamnogalacturonan II, while mannose-containing polymers are present in high amounts. In bryophytes, cell wall composition generally resembles that in charophytes, while the cell walls of lycophytes and monilophytes contain typical "angiosperm" hemicelluloses and pectins, and can be secondarily lignified. Seedless plants contain hydroxyphenyl and guaiacyl lignins while *Selaginella moellendorffii* possesses an angiosperm-type syringyl lignin, an example of convergent evolution (Weng et al., 2008; Weng and Chapple, 2010). Homogalacturonan, an important component of the pectin enclosing PD in angiosperms, in *Selaginella moellendorffii* shows a recalcitrance to the typical extraction methods which might indicate the presence of specific modifications (Harholt et al., 2012). A special kind of mixed beta-1-3- and beta-1-4-linked glucans (MLG) typical of Poaceae has been found in *Selaginella moellendorffii* which is another example of convergent evolution, since in *Selaginella moellendorffii*, MLG is synthesized by enzymes which are non-homologous to those in angiosperms (Harholt et al., 2012). Altogether, the assortment of hemicellulose and pectins in cell walls of seedless plants is not the same as in seed plants. Contrarily, callose, which is, perhaps, the most important regulator of PD diameter in angiosperms, has been found in characean algae and throughout all embryophyte taxa (Fangel et al., 2012). Another potentially important point for PD functions is the fact that seedless plants have an evolutionary ancient complement of sphingolipids (which, however, has been also detected in Poales as a further example of convergent evolution; Nurul Islam et al., 2012), as sphingolipids represent an important component of lipid rafts in plants (Mongrand et al.,





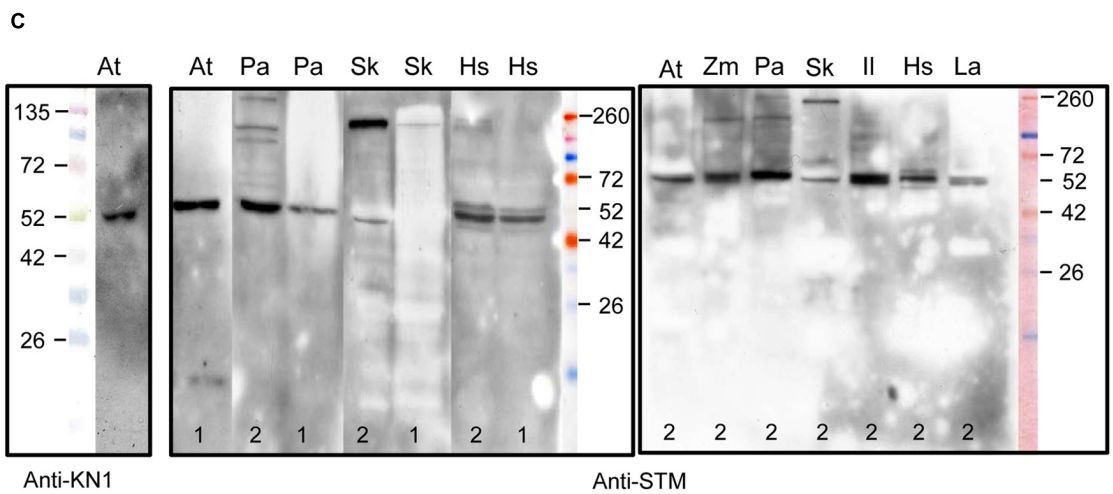


FIGURE 1 | Continued

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(A) Occurrence of primary and secondary plasmodesmata (PD) in sporophytes throughout the main groups of embryophytes (Kenrick and Crane, 1997; Judd et al., 2002). Stars indicate the presence of secondary PD; different colors of stars stand for independent evolutionary origins. **(B)** Schematized sections of three types of the SAM symplasmic structure in sporophytes of land plants. Line drawings are based on original unpublished data of the authors for *Selaginella kraussiana* **(A)**, *Huperzia selago* **(B)** and *Syringa vulgaris* **(C)**. Different colors in **(A)** mark successive segments of the single apical cell (merophytes) and their derivatives. Single and plural apical initials in **(A)** and **(B)** are marked with red color. In **(C)** red color marks cell layers of the tunica. Primary PD are symbolized by dashes, secondary PD by crosses. The numbers of dashes and crosses per cell wall do not reflect actual PD density but roughly (in limits of one order of magnitude) correspond to the original unpublished data of the authors for *Selaginella kraussiana* and *Huperzia selago*, and to the data of Imaichi and Hiratsuka (2007) for Lycopodiaceae, Selaginellaceae, and Isoetaceae species. **(A)** Lineage-specific networks of primary PD in ferns and lycophytes from the Selaginellaceae family. **(B)** Interface-specific network of primary and secondary PD in lycophytes from the Lycopodiaceae and Isoetaceae families; primary and secondary PD seem to be distributed randomly. **(C)** Interface-specific network of primary and secondary PD in angiosperms; basal periclinal walls of tunica cells form long-persisting cellular boundaries equipped exclusively with specialized (secondary) PD. **(C)** Specificity of an antibody raised against a peptide region of the *Arabidopsis thaliana* shoot meristemless (STM) protein to KNOX homologs from a range of embryophyte taxa (http://www.agrisera.com/en/artiklar/stm-homeobox-protein-shoot-meristemless.html). Immunoblots of 10 μg of total protein extract per slot are shown for *A. thaliana* 2-week-old seedlings (At), *Picea abies* shoot apices (Pa), *Huperzia selago* shoot apices (Hs), *Selaginella kraussiana* shoot apices (Sk), *Lycopodium annotinum* shoot apices (La), *Isoetes lacustris* shoot apices (Il), and *Zea mays* shoot apices (Zm). Proteins were extracted either according to Conlon and Salter (2007; 1) or Carpentier et al. (2005; 2). Details of protein separation, blotting and development of the blots are published on the Agrisera website (see above). An anti-KN1 antibody raised against *Z. mays* KNOTTED1 protein (Smith et al., 1992) was used with protein extracts from Arabidopsis seedlings. Primary antibodies were diluted 1:5000. In *Arabidopsis*, both antibodies recognize a single band of about 52 kDa which is running higher than the predicted molecular weight (MW) of STM protein (42.7 kDa). Similar results were obtained with anti-KN1 antibody on immunoblots with *Z. mays* protein extracts: while the predicted MW for ZmKNOTTED1 is 39.0 kDa, single bands recognized by the antibody showed an apparent MW of 42 kDa (Smith et al., 1992), 50 kDa (Harrison et al., 2005), or of 47 kDa (Nowak et al., 2011). Predicted molecular masses of *Arabidopsis* STM and its known homologs: *A. thaliana* STM (AEE33958.1), 42.7 kDa; *Picea abies* homeobox protein (AAC84001.1), 48.6 kDa; *Selaginella kraussiana* SkKNOX1 (AAW62517.1), 51.4 kDa. For the other species on these blots, the sequences of the homologs are unknown.

2010), structures which recently were speculated to be involved in PD function (Tilsner et al., 2011).

Another peculiarity which might have been expected to have some impact on PD formation is monoplastidy of some cells in bryophytes and in the lycophyte genera *Selaginella* and *Isoetes*, including cells in their apical meristems (Brown and Lemmon, 1984, 1990). Monoplastidy introduces some important changes into the mechanism of mitosis: to ensure that each sister cell receives a plastid, an unusual plastid-based mitotic spindle is formed during monoplastidic divisions. This changes the geometry of the microtubules in the mitotic spindle as compared to the situation in dividing polyplastidic cells, in that microtubules are not uniformly distributed over the region of cell plate formation and lay perpendicular to it, but are bound on two foci at the plastid. Thus, it might be expected that the pattern of primary PD laid along these microtubules also changes accordingly. However, so far the formation of phragmoplasts and of primary PD has not been reported to be influenced by the involvement of plastids in cell division in these plants. Generally, the structure of PD in seedless plants was found to be similar to that in seed plants; there is, to our knowledge, only one exception, namely, the unique structure of expanded PD in angle and apical meristems of *Selaginella willdenovii* (Wochok and Clayton, 1976).

A prominent feature of PD in angiosperms is their participation in the cell-to-cell spread of viruses. Interestingly, there are very few, altogether seven, reports on virus infection and spread in seedless vascular plants (Valverde and Sabanadzovic, 2009 and references therein; Scheets et al., 2011 and references therein), including a report on a new previously unknown RNA virus found in the fern *Cyrtomium falcatum* which was assigned to a new taxon named Pteridovirus (Valverde and Sabanadzovic, 2009). Infected plants of *Cyrtomium falcatum* showed visible symptoms indicative of spread of this virus via PD; the infection was shown to be transmitted from infected to healthy ferns by grafting and from spore to spore, while attempts to transmit the virus to several angiosperm species by injury failed (Valverde and Sabanadzovic, 2009). The extremely rare occurrence of viral diseases in seedless plants, as well as the unusual properties of this virus which is probably limited to fern hosts only, might indicate important differences in the organization of PD and the regulation of cell-to-cell transport of macromolecules in seedless plants. Another indication comes from the analysis of genome in *Selaginella moellendorffii* (Banks et al., 2011). First, the trans-acting small interfering RNA (tasiRNA) pathway is lacking in this species. tasiRNA represent silencing signals known to act non-cell-autonomously and spread locally over about a 10–15-cell-distance in angiosperms (reviewed by Hyun et al., 2011). Second, the proportion of the small RNA of 24 nt in length, which are known to spread systemically over long distances, is very low in *Selaginella moellendorffii*. Altogether, these data might indicate that in ancient taxa of vascular plants, the ability of PD to mediate the transport of viral and endogenous RNAs is much lower than in angiosperms.

Information on the regulation of PD biogenesis and of transport through PD is yet scarce. Transport through PD is regulated by turgor pressure differentials in tobacco leaf trichomes (Oparka and Prior, 1992) and in *Chara corallina* (Ding and Tazawa, 1989), and has been proposed to be regulated in a similar manner in PD between mesophyll and phloem companion cells (Voitsekhovskaja et al., 2006). PD SEL and the spatial distribution of complex PD in *Arabidopsis* leaves were recently suggested to be the subject of osmotic regulation (Fitzgibbon et al., 2013). Although there are indications for transport of hormones through PD, few data on the hormonal regulation of PD function are available for angiosperms. Gibberellins were shown to influence callose turnover at PD through up-regulation of glucan hydrolase family genes, leading to the reopening of signal conduits and to the release of dormancy after chilling in *Populus* (Rinne et al., 2011). Salicylic acid (SA) has been found to accelerate the formation of complex PD up to threefold in *Arabidopsis* leaves (Fitzgibbon et al., 2013). As complex PD are characterized by a reduced SEL, their enhanced formation in response to SA might be a part of the response to pathogen stress that reduces the non-selective movement of pathogen-induced macromolecules or viruses between host cells. Another aspect of the role of SA in the innate immune response involves the

induction of callose accumulation around PD via up-regulation of specific callose synthases (Wang et al., 2013).

Cytokinins, auxins, abscisic acid (ABA), brassinosteroids, and ethylene are ancient plant hormones already present in algae, but the responses to these hormonal signals evolved to a different extent in various embryophyte taxa (Paponov et al., 2009; Pils and Heyl, 2009; Prigge and Bezanilla, 2010; Kutschera and Wang, 2012; McAdam and Brodribb, 2012; Yasumura et al., 2012). Gibberellins and jasmonates evolved later. Components of the jasmonate biosynthesis are present in *P. patens* but jasmonate is not synthesized (Stumpe et al., 2010). No data on the jasmonate biosynthesis pathway in lycophytes are available, but Li et al. (2012) observed the production of volatile terpenes (induced by jasmonate in seed plants) in *Selaginella moellendorffii* in response to fungal elicitors, similar to seed plants, although the enzymes of the pathway are partially encoded by unique genes not homologous to their angiosperm equivalents. Key components of gibberellic acid signaling are present in the genome of *Selaginella moellendorffii* but not of *P. patens* (Schwechheimer and Willige, 2009). In angiosperms, cytokinins were reported to promote secondary PD formation (Ormenese et al., 2000, 2006), while the ratio of ABA to cytokinins was shown to influence the ultrastructure of PD (Botha and Cross, 2000). As only primary PD have been found in bryophytes, in the lycophyte family Selaginellacae, and in ferns, while secondary PD in the Lycopodiaceae and Isoetaceae families of the lycophytes evolved independently from those in seed plants (see below), it would be interesting to investigate the effects of cytokinin on PD formation and function in these groups.

A recently discovered fascinating facet is the regulation of PD formation and transport via PD in angiosperms by reactive oxygen species and plastid signals (Benitez-Alfonso et al., 2009; Burch-Smith et al., 2011; Burch-Smith and Zambryski, 2012; Stonebloom et al., 2012). One example of this is the regulation of PD in *Arabidopsis* roots by different levels of hydrogen peroxide (Rutschow et al., 2011). This has never been investigated in ancient plant taxa.

THE EVOLUTION OF SECONDARY PD AND SHOOT APICAL MERISTEM ORGANIZATION IN LAND PLANTS

Although both primary and secondary PD occur in angiosperms, a special role in transport of developmental regulators, signals, and viruses has been assigned to secondary PD (Ding et al., 1992, 1993; Ding and Lucas, 1996). Ding et al. (1993) hypothesized that secondary PD differ from primary PD in being able to transport developmental signals. Ding et al. (1992) found that PD between mesophyll cells of the leaf, which are primary PD by origin with secondary modifications added later on, differ from the originally secondary PD at the interface between phloem companion cells and bundle sheath cells in that tobacco mosaic virus movement protein (TMV-MP) gates the former but not the latter PD (Ding et al., 1992; but see Crawford and Zambryski, 2001). Non-cell-autonomous transcription factors are synthesized in one cell and travel through PD to act in another cell. Kim et al. (2003) studied in transgenic *Arabidopsis* the traffic of fusions of GFP with KN1, the non-cell-autonomous transcription factor KNOTTED1 from *Zea mays* (Hake and Freeling, 1986) which functions in the shoot apical meristem (SAM). They found that GFP-KN1 is able to traffic from the L1 into the L2 meristematic layer in the SAM but not further, and in mature leaves, GFP-KN1 traffics from mesophyll cells into the epidermis (produced by the L2 and L1 SAM layers, respectively) but not inversely. Interestingly, in maize SAMs, KNOTTED1 traffics from the L2 into the L1. Since PD between L1 and L2 are secondary in origin, these data seem to suggest that secondary PD in angiosperms are specifically equipped to enable the transport of non-cell-autonomous developmental regulators like KNOTTED1. On the other hand, the observations that a number of transcription factors, e.g., CPC and SHR (Kurata et al., 2005; Rim et al., 2011), can move also between cells of the same lineage connected via primary PD (e.g., trichoblasts and atrichoblasts or endodermis and cortex, respectively), seem to contradict this suggestion.

Studies on fern gametophytes and on the apical meristems of fern sporophyte roots and shoots have shown that here, all PD arise exclusively during cytokinesis, i.e., are primary (Gunning, 1978; Gunning et al., 1978; Tilney et al., 1990; Imaichi and Hiratsuka, 2007). Strikingly, the (in)ability to form secondary PD is reflected by the symplasmic organization of the SAM. In ferns, SAMs possess a single initial, also named the apical cell, which produces merophytes in a highly regular fashion. All cell walls observed within the SAM are formed recently, as the merophytes are rapidly displaced by newly formed ones. Ultrastructural and developmental studies in ferns revealed several mechanisms regulating PD numbers between the cells. First, "an abrupt increase in plasmodesmatal number occurs in the cell wall that is destined to construct one side of the future apical cell before that apical cell appears" (Tilney et al., 1990). Second, in the course of merophyte cell divisions, the number of PD in the newly formed cell walls is determined so as to match the PD density of the already existing cell walls, while taking into "consideration" the future expansion of this newly formed cell wall (Gunning, 1978). Third, before the cessation of growth, PD numbers decrease in the walls of the last subapical cells formed from the senescent apical cell (Gunning, 1978). Thus, PD amount and density in cell walls of mature cells depend on the initial amount of PD laid by the apical cell during its division, on the extent of cell wall expansion, on the total number of cell divisions and on eventual loss by occlusion. This results in a PD density gradient starting from the apical cell, where the PD densities are among the highest reported thus far (Cooke et al., 1996). Moreover, the potential to grow indefinitely might be limited by the numbers of PD between the cells in ferns (Gunning, 1978). In seed plants, SAMs possess multiple initials organized in several cell layers. The periclinal cell walls between the layers persist within the meristem during its life, and sometimes can even be traced back to the protoderm of the embryo (Cooke et al., 1996). As the cells in SAMs of seed plants can form secondary PD, there are no visible gradients of PD density. On the basis of the analyses of PD densities and distribution patterns in fern and seed plant root and SAMs, Cooke et al. (1996) proposed two main types of PD network organization in embryophytes: the lineage-specific network of primary PD which connect cells of the same lineages and is found in ferns, and the interface-specific network of primary and secondary PD which can connect any adjacent cells and is typical for seed plants. The type of PD network strongly correlates with the organization of the SAM in that SAMs with single initials have the lineage-specific network of primary PD and those

with plural initials have the interface specific network of primary and secondary PD, respectively (Cooke et al., 1996).

It is generally accepted that embryophytes evolved from a characean ancestor forming exclusively primary PD (Cook et al., 1998). At what time did the ability to form secondary PD appear in the evolution? In Lycopodiophyta, a sister group to other vascular plants, SAMs with single initials and PD density gradients are found in representatives of the Selaginellaceae family, while SAMs with multiple initials and evenly distributed primary and secondary PD are found in representatives of the Isoetaceae and the Lycopodiaceae families (Imaichi and Hiratsuka, 2007). This raises the question whether the ability to form secondary PD appeared independently in seed plants and some Lycopodiophyta, or was lost in ferns. PD formation and distribution in bryophytes follow the fern pattern both in the gametophyte and the sporophyte SAMs, which indicates an independent origin of secondary PD in Lycopodiophyta (Ligrone and Duckett, 1998; Mansouri, 2012; **Figure 1A**). However, observations on *Sphagnum palustre* leaflet development showed that PD density did not decrease in the course of equal cell divisions, and even increased after the first unequal division (Schnepf and Sych, 1983). This might suggest that the mechanism for secondary PD development evolved in bryophytes, although as a homoplasy, as it occurs only in the gametophyte generation.

Formation of secondary PD is considered necessary for successful graft unions. Interestingly, there is only one record of grafting lycophytes, namely from Daniel (1901) for *Selaginella arborea* (now *Selaginella willdenovii*), and to our knowledge grafting of lycophytes has never been reported since. However, grafting is indeed possible in ferns (e.g., Valverde and Sabanadzovic, 2009), indicating that, in spite of the absence of secondary PD in their tissues, ferns, but probably not lycophytes, can form secondary PD in special cases.

These data indicate that the cell boundaries in SAMs of "fern" and of "seed plant" types, respectively, are specified by different mechanisms, raising the question whether transport of developmental regulators via PD occurs similarly in SAMs of seed plants, in the "fern" type SAMs and in the "seed plant" type SAMs of Lycopodiaceae containing secondary PD of independent evolutionary origin. In SAMs of the fern type, the informational exchange via PD between cells and merophytes seems to be only possible within cell lineages. The fact that cell walls are rapidly displaced from the SAM might make it impossible to establish a long-persisting boundary equipped with specialized PD between meristematic domains, like those between the L1, L2, and L3 cell layers in angiosperms SAMs. The absence of secondary PD raises the question whether primary PD are able to exert efficient control over selective traffic of developmental signals in "fern" type SAMs. The SAMs of "seed plant" type in Lycopodiophyta are organized similarly to those in angiosperms in that they contain both primary and secondary PD, but the independent evolutionary origin of secondary PD makes it probable that the transport via these PD is regulated by different mechanisms. At the same time, contrarily to the SAMs of seed plants, the SAMs of Lycopodiaceae seem to contain no long-persisting boundaries between meristematic domains. In **Figure 1B**, we show schematized depictions of the three types of the SAMs organization.

LYCOPHYTES AS THE MODELS TO STUDY EVOLUTION OF NON-CELL-AUTONOMOUS TRANSPORT OF DEVELOPMENTAL REGULATORS IN VASCULAR PLANTS

Lycophyte species belonging to the Selaginellaceae and Lycopodiaceae/Isoetaceae, respectively, seem to be the most interesting models for studies of non-cell-autonomous transport in ancient plant taxa, in our opinion, because they would allow to analyze the trafficking of developmental regulators in SAMs with two most contrasting symplasmic organization within the same taxon Lycopodiophyta. Another strong argument for the studies of Lycopodiophyta is the current availability of the only genome from a seedless vascular plant, namely that of *Selaginella moellendorffii*. Nevertheless, in the future, studies of PD function in the development in all taxa of land plants will be necessary to complete the evolutionary view of the establishment of this phenomenon. In angiosperms, the best studied example of non-cell-autonomous regulators in SAMs are KNOX transcription factors (Jackson, 2005). Recently, Chen et al. (2013) could show on the basis of trichome rescue assays the non-cell autonomous mode of action of KNOX proteins from the moss *P. patens*, but not from the unicellular alga *Chlamydomonas*, and concluded that the ability to efficiently traffic through PD may have been acquired early in the evolution of land plants for KNOX homeodomain proteins. In bryophytes, KNOX genes play a role in sporophyte development (Sakakibara et al., 2008). The functions of KNOX genes are conserved among lycophytes, ferns and angiosperms (Harrison et al., 2005). Comparison of the cellular distribution patterns of the gene transcripts and proteins, respectively, can give an insight into the non-cell-autonomy of the KNOX protein homologs in seedless plants (Jackson, 2002). KNOX gene homologs have been cloned in some seedless vascular plant species, and the localization of their transcripts and encoded proteins have been performed in several cases (**Table 1**). Unfortunately, none of the studies compared patterns of transcript and protein localization in the same species at the cellular and tissue level, so at present, no conclusions can be drawn about the non-cell-autonomy of the KNOX proteins in seedless plants. For such studies, obtaining high-resolution cytological pictures, which is rather challenging for SAMs with a single initial, may become a critical point. Another important aspect is the availability of the antibodies able to specifically recognize KNOX homologs in SAMs of seedless plants. We analyzed the specificity of several antibodies raised against KN1 protein from *Z. mays* (Smith et al., 1992) and against shoot meristemless (STM) from *Arabidopsis* (Agrisera, Sweden) on Western blots with total protein extracts from representatives of a range of embryophyte taxa using two different extraction methods (**Figure 1C**). The antibodies recognized, depending on the species and the extraction method, one to several KNOX protein homologs, showing that they can be used for immunohistochemical studies in lycophytes. Transcriptome analyses of shoots of Lycopodiaceae species, along with the available data on *Selaginella moellendorffii KNOX* gene homologs, may provide a tool for analyses of KNOX gene transcript localization in SAMs of lycophytes to be compared with the localization of the proteins. Along with *Selaginella moellendorffii*, *Selaginella kraussiana* is another convenient model for such studies because KNOX genes have been cloned and studied in this species (Harrison et al., 2005). *Huperzia selago* is a representative

Table 1 | Localization on KNOX gene products (mRNA, proteins) in seedless vascular plants.

Species	Product of the *KNOX* gene	Localization of the *KNOX* gene product	Reference
Polypodiophyta			
Ceratopteris richardii		Class 1 *KNOX* genes	Sano et al. (2005)
	CrKNOX1 mRNA	SAM: single initial, surface initial derivatives; leaf primordia; procambium	
	CrKNOX2 mRNA	SAM: single initial, surface initial derivatives; leaf primordia; procambium	
		Class 2 *KNOX* genes	
	CrKNOX3	No data	
Osmunda regalis	KNOX protein homolog	SAM: periferal meristem cells, subsurface meristem cells; leaf primordia; procambium	Harrison et al. (2005)
Anogramma chaeophylla	KNOX protein homolog	SAM; leaf primordia; procambium	Bharathan et al. (2002)
Lycopodiophyta			
Selaginella kraussiana		Class 1 *KNOX* genes	Harrison et al. (2005)
	SkKNOX1 mRNA	SAM: subsurface meristem cells	
	SkKNOX2 mRNA	Internodal regions of the shoot	
		Class 2 *KNOX* genes	
	SkKNOX3	No data	
Selaginella uncinata		Class 1 *KNOX* genes	Kawai et al. (2010)
	SuKNOX1 mRNA	SAM cells including single initial	
		Class 2 *KNOX* genes	
	SuKNOX2	No data	

of the Lycopodiaceae family which is characterized by a highly ordered structure of a "seed plant" type SAM with several rectangular apical initials and vigorously dichotomously branching shoots with relatively big apices that can yield sufficient amounts of RNA for molecular studies (Romanova et al., 2010). This species could provide a Lycopodiaceae model to supplement the studies on Selaginellaceae species.

In conclusion, structural data on SAM organization and -function in seedless vascular plants, the presence of two contrasting types of PD networks in lycophytes, and the availability of the genome sequence of *Selaginella moellendorffii*, make functional studies of PD in lycophytes an interesting option which will help to understand the role of intercellular transport via PD in the morphogenesis of vascular plant taxa with different phylogenetic history. The present, yet scarce and indirect information suggests that the ability of the PD to mediate cell-to-cell transport of macromolecules in ancient taxa of vascular plants might differ from that in angiosperms. The comparative study of cellular patterns of localization of transcripts and protein homologs of the known non-cell-autonomous developmental regulators in the representatives of the Selaginellaceae and Lycopodiaceae/Isoetaceae can provide the first evidence in testing this hypothesis.

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Importance of the hexagonal lipid phase in biological membrane organization

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Domains are present in every natural membrane. They are characterized by a distinctive protein and/or lipid composition. Their size is highly variable from the nano- to the micrometer scale. The domains confer specific properties to the membrane leading to original structure and function. The determinants leading to domain organization are therefore important but remain obscure. This review presents how the ability of lipids to organize into hexagonal II or lamellar phases can promote particular local structures within membranes. Since biological membranes are composed of a mixture of lipids, each with distinctive biophysical properties, lateral and transversal sorting of lipids can promote creation of domains inside the membrane through local modulation of the lipid phase. Lipid biophysical properties have been characterized for long based on *in vitro* analyses using non-natural lipid molecules; their re-examinations using natural lipids might open interesting perspectives on membrane architecture occurring *in vivo* in various cellular and physiological contexts.

Keywords: glycerolipid, lipid bilayers, hexagonal phase, membrane domains, lipid phase

Domains are present in every natural membrane. They are characterized by a distinctive protein and/or lipid composition and they confer specific properties to the membrane leading to original structure and function. Plasmodesmata, a highly specialized membrane organization that connects two plant cells, involves membrane domains. This typical plant structure is composed of two membranes: the plasma membrane and the desmotubule, a narrow tube in continuity with the endoplasmic reticulum (ER). Membrane domains described as lipid raft were found in the plasma membrane of plasmodesmata and might be involved in plasmodesmata scaffolding but nothing is known about the lipid organization of the desmotubule. The diameter of the membrane desmotubule is between 10 and 15 nm, which is highly constricted for a bilayer (Tilsner et al., 2011). Continuity of the membrane and of the luminal space between reticulum and desmotubule is now clearly established (Tilsner et al., 2011) but the organization of the desmotubule membrane as a bilayer has never been demonstrated. The presence of non-bilayer phase in desmotubule as a hypothesis may provide a new angle for desmotubule model establishment. This review presents how the ability of lipids to organize into non-lamellar phases, particularly hexagonal II (HII) phase, can promote specific local structures within membranes.

Works on lipid membrane organization were first done by physical chemistry using synthetic lipids and model membranes but they gave us the premises for apprehending the biology of cell membrane structure. According to the Singer and Nicholson's model (Singer and Nicolson, 1972), cell membranes are viewed as proteins embedded in a lipid matrix. This so-called mosaic fluid model includes two basic postulates referring to the "lipid phase" state – *liquid crystalline* and *bilayer*, both of which are of vital importance for the proper functioning of membranes. In *in vitro* systems, aqueous dispersions of lipids are however able to form a large variety of other phases such as non-liquid crystalline and non-bilayer phases. These "solid phases" (also called "gel phases") are favored by low temperature and long and saturated fatty acid chains. They were mainly characterized on model membranes of saturated phosphatidylcholine (PC; for example, see Mason, 1998). However, natural lipids are mostly unsaturated and organisms adapt their fatty acid composition to the environment to prevent the formation of gel phases. Moreover, even though gel phase domains were detected in biological membranes in very specific cases such as the myelin sheath (Ruocco and Shipley, 1984) or in the stratum corneum (Norlen, 2001b), most biological membranes are organized in liquid phase. Therefore gel phases will not be described further in this review.

Since the mosaic fluid model largely neglected the possibility that lipids are not randomly distributed in the bilayer and also understated the degree of local order that can be generated in membranes, this model was soon enriched with the introduction of the membrane domain concept (for a review on membrane model history, see Edidin, 2003). The domains, identified at first *in vitro* in model membranes (Jain and White, 1977), were further confirmed *in vivo* with the raft concept (Simons and Ikonen, 1997) and defined as patches of lipids with composition and physical state that differed from the average.

The raft domains that involve also sterols and sphingolipids and correspond to patches of lipids, in a liquid ordered phase, within a matrix in a liquid disordered phase, have been referenced in depth in different reviews (Bagatolli et al., 2010; Simons and Sampaio, 2011) and will not be described further here. This review will consider more specifically domains resulting from modification of glycerolipid biophysical organization since glycerolipids represent the main constituent of the membrane lipid matrix. We shall focus here on the non-bilayer organization that can adopt glycerolipids, their biophysical properties, and their impact on membrane biology, since this topic is rarely raised in recent biological literature.

Membrane glycerolipids are a category of amphiphilic molecules having a 3-carbon glycerol scaffold (each carbon is numbered following the stereospecific numbering nomenclature *sn*-1, *sn*-2, *sn*-3), harboring one or two hydrophobic acyl chains esterified at positions *sn*-1 and *sn*-2, and a hydrophilic polar head at position *sn*-3. Glycerolipids can be separated into two classes in function of their polar head: phospholipids that contain a phosphorous atom and non-phosphorous glycolipids. Major membrane phospholipids found in prokaryotes and eukaryotes are PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) also called cardiolipin, phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). Major glycolipids are monogalactosyldiacylglycerol (MGDG), monoglucosyldiacylglycerol (MGlcDG), digalactosyldiacylglycerol (DGDG), diglucosyldiacylglycerol (DGlcDG), and sulfoquinovosediacylglycerol (SQDG). Physical studies showed that the aqueous dispersions of glycerolipid do not always spontaneously form lipid bilayers as it was guessed at first (Gorter and Grendel, 1925). Indeed, the size of the polar head by comparison of the hydrophobic acyl-glycerol backbone affects lipid behavior in aqueous dispersions (**Figure 1**). By convention, large negative curvature lipids such as MGDG, MGlcDG, PE, DPG, PS, and PA tend to form HII phase or cubic phase, large positive curvature lipids such as lysolipids form hexagonal I (HI) phase whereas small curvature lipids such as DGDG, DGlcDG, SQDG, PC, PG, and PI form lamellar phase, corresponding to the classical bilayer (Shipley et al., 1973; Seddon, 1990; Hansbro et al., 1992; Vikstrom et al., 1999). Bilayers create a planar structure whereas HI phase forms micellar tubules with the polar head on the outside of the tubules and HII phase forms inverted tubules, with the fatty acyl chains pointing toward the outside of tubules and the polar head groups toward the center establishing an aqueous channel (**Figure 1**).

However, within a class of lipids, fatty acids can also influence the lipid architecture; the effect of increasing chain length and of unsaturation number is expected to favor in general the

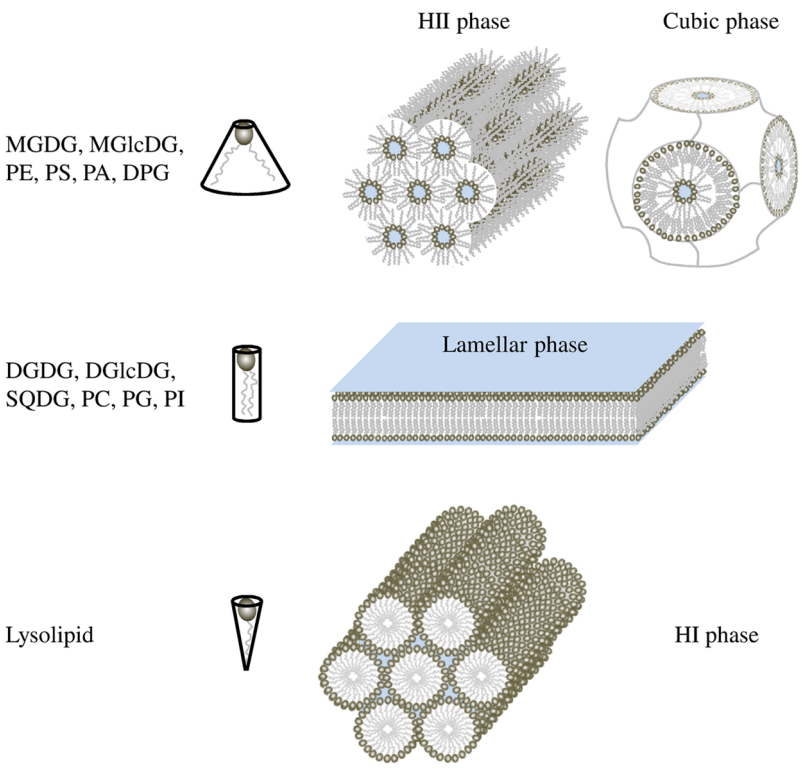

FIGURE 1 | Shape structure concept of lipid polymorphism. Lipids with a small polar head have a molecular shape that resembles a truncated cone. They induce a negative curvature strain and favor the organization of membranes into inverted micelles (HII phases) or cubic (bicontinuous) structures. Lipids with a bulky polar head and only one acyl chain have a molecular shape similar to an inverted cone and induce a positive curvature strain in membranes. They favor the formation of tubular (HI) or spheric micelles. Lipids that have similar cross-sectional areas for the polar head and hydrophobic region look like cylinders. They form lamellar phases, with no curvature strain.

formation of HII phase. For instance, saturated PE form a lamellar phase whereas unsaturated PE form an HII phase (for a review, see Seddon, 1990). Furthermore, HII forming lipids are able to switch from a HII phase to a lamellar phase sometimes through an intermediate cubic phase (**Figure 1**) by lowering the temperature (Tenchov and Koynova, 2012). Proteins and pigments might also be involved in cubic phase formation (Wang and Quinn, 1999; Almsherqi et al., 2006; Tenchov et al., 2013). All these phase transitions are spontaneous and reversible (Siegel and Tenchov, 2008).

Biological membranes of course contain complex mixtures of lipids, and so it is of great importance to understand the polymorphic phase behavior of such mixtures in well-defined model systems. The use of synthetic lipid mixture and the development of techniques, such as electron microscopy, nuclear magnetic resonance (NMR), X-ray, and neutron diffraction, helped a lot to characterize the parameters that trigger the transition from lamellar phase toward HII phase. Lipid membrane composition, hydration, pH, and presence of cations contribute to lipid organization. For example, an equimolar mixture of PE and PC at low hydration pressure is organized in HII phase whereas at high hydration pressure it adopts a bilayer conformation (Ding et al., 2005). Lowering the pH induces lamellar toward HII transition phase in charged phospholipid system such as PS and PA (Seddon, 1990). Furthermore, transition of DPG from lamellar phase to HII phase is induced either upon lowering pH to below 2.8, or upon increasing NaCl concentration to above 1.6 M at pH 7 (Seddon et al., 1983).

The question now is: Do these structures occur *in vivo*? Most natural membranes are composed of two main glycerolipids: a bilayer forming lipid and a HII forming lipid, respectively the couple PC/PE in yeast and animal cells, PG/PE in *Escherichia coli* and *Bacillus subtilis*, DGlcDG/MGlcDG in *Acholeplasma laidlawii*, and DGDG/MGDG in plants. At a microscopic level, HII structure have been observed in the ER of the retinal pigment epithelium (Yorke and Dickson, 1985) and of the plasma membrane of bladder epithelium (Hicks and Ketterer, 1970). Cubic structures (**Figure 1**) that are dependent of non-bilayer forming lipids (Siegel, 1999) are also detected in the ER of epidermal keratinocytes (Norlen, 2001a) and in prolamellar bodies in etioplasts (Williams et al., 1998; Gunning, 2001). Furthermore, highly curved membranes like tubules of ER network (Griffing, 2010), the inner mitochondrial membrane (Van Venetie and Verkleij, 1982), or thylakoid grana margins (Murphy, 1982) are thought to be favored by an enrichment in HII forming lipid. At a smaller scale, inverted micellar structures (**Figure 2**) have been proposed to explain structures observed by NMR in some bilayer systems (for a review see Siegel, 1984).

What is the function of these structures? These structures may be of high importance for some enzyme activities. Enzymes like the calcium pump in the sarcoplasmic reticulum (Yeagle, 1989), the CTP: phosphocholine cytidylyltransferase (Attard et al., 2000) or the violaxanthin de-epoxidase in the thylakoids (Latowski et al., 2004) have an activity dependent of HII structures. Membrane anchoring of some proteins like G-proteins and phosphokinases C is enhanced by HII phase (Escriba et al., 1997; Vogler et al., 2004). Membrane fusion and fission events seem also to be dependent on the presence of non-bilayer forming lipids (for a review, see Burger, 2000). It has been shown that lipid bilayers can fuse in the complete absence of proteins even if membrane fusion is regulated *in vivo* by specialized proteins. Membrane fusion between phospholipid bilayers can be induced by the HII lipids, PA, and PS, in conjunction with Ca^{2+} (for a review see Papahadjopoulos et al., 1990) or by dehydration, that drives bilayers into very close contact (Yang and Huang, 2003). All actual models (Chakraborty et al., 2012; Hamilton et al., 2012; Colpitts et al., 2013) for the membrane fusion process share at least one intermediate structure called the fusion stalk (**Figure 2**; Markin et al., 1984). Stalk formation is promoted by an HII forming lipid like PE whereas it is inhibited by an HI forming lipid like lysoPC (Chernomordik and Kozlov, 2008). The stalk structure is also an intermediate in the lamellar/HII phase transition (**Figure 2**) and was observed for the first time in a mixture of PE/PC upon dehydration (Yang and Huang, 2002). Parameters affecting the lamellar/HII phase transition can probably be considered also as fusion parameters and biophysical studies of this phase transition, such as calculation of the energy needed for the process, is starting to give a lot of insights on membrane fusion mechanism (Kozlovsky et al., 2004; Pan et al., 2006).

For the preservation of cell structure and compartmentalization, the membrane needs to be in a lamellar phase but, for membrane architecture and for some enzyme activities, HII phase domains must be present. It was shown that bacteria cells are able to keep the membrane lipids in a "window" between lamellar phases and HII phases. For example, *E. coli* or *A. laidlawii* maintain a balance between HII forming lipids and bilayer forming lipids by adjusting the composition of the polar head group (*A. laidlawii*) or the acyl chains (*E. coli*; Lindblom et al., 2002). This lead to the hypothesis that biomembranes homeostatically adjust their intrinsic curvatures to maintain a constant net spontaneous curvature in each leaflet of the bilayer (Gruner, 1985). Activation of the CTP: phosphocholine cytidylyltransferase by HII phase might be a key factor for this kind of adaptability (Attard et al., 2000). On this model, it was postulated that several enzymes involved in lipid biosynthesis could also be regulated by membrane stored curvature elastic energy. Kinetic simulations of the eukaryotic lipid biosynthetic pathway were used to show how this elastic energy was homeostatically maintained through a HII/bilayer ratio control mechanism (Alley et al., 2008; Beard et al., 2008) similarly to what was proposed for *A. laidlawii* (Vikstrom et al., 2000).

In conclusion, the biophysical properties of lipids have been characterized for long based on *in vitro* analyses using non-natural lipid molecules; their re-examinations using natural lipids might open interesting perspectives how membrane structure organizations occur *in vivo* in various cellular and physiological contexts, like in plasmodesmata. This might comfort the theory that cells adjust their membrane lipid composition in response to perturbations in order to maintain bilayer stability, but keeping the bilayer close to a point of instability, where a confined transformation to some non-bilayer structure would tend to occur. The mechanisms "sensing" the physical state of lipids and regulating the lipid biosynthetic pathways accordingly are unknown.

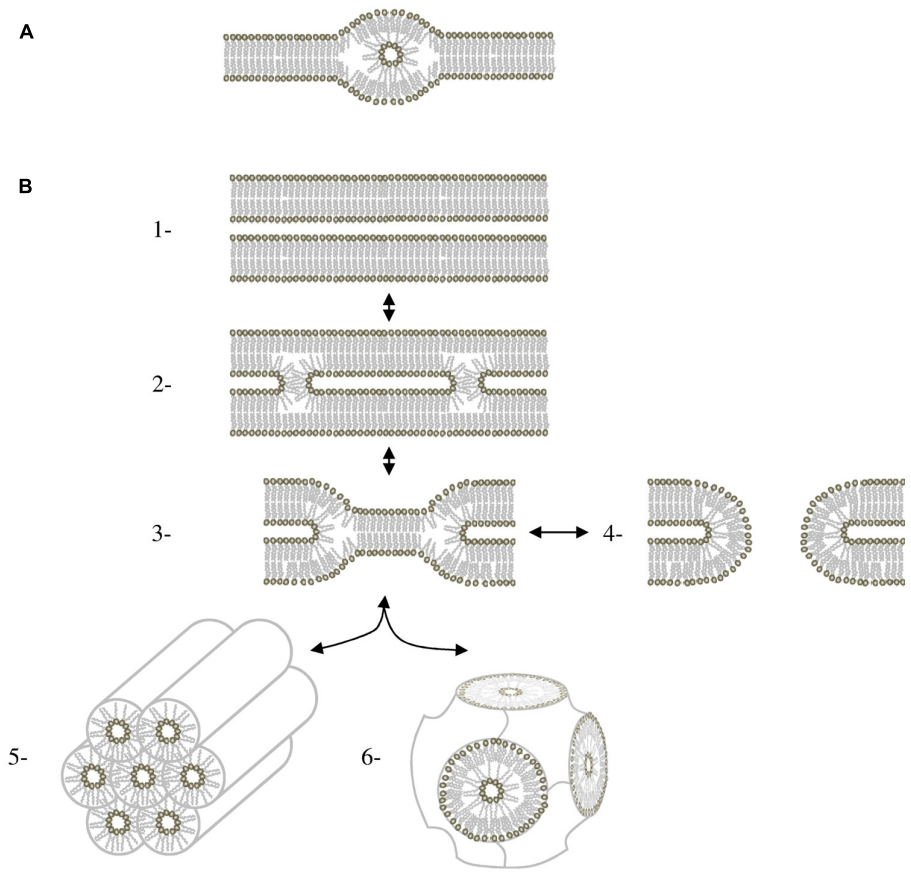

FIGURE 2 | HII phase in bilayers. (A) Lipidic particle as described in (Siegel, 1984). **(B)** Mechanisms of membrane fusion involving HII *via* the stalk intermediate. (1) Apposition of two bilayers. (2) Stalk. The stalk is cylindrically symmetrical. (3) Hemifusion intermediate. It can form two different types of structures. If the bilayer diaphragm in the middle of the hemifusion intermediate ruptures, it forms a fusion pore (4) If fusion pores accumulate in sufficient numbers, they can rearrange to form a cubic phase (5) For systems close to the lamellar/HII phase boundary, hemifusion intermediates can also aggregate to form HII phase (6) Figure adapted from Siegel (1999).

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