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APPLICATION OF PROTEOMICS FOR IMPROVEMENT IN CROP PROTECTION/ARTIFICIAL REGULATION

Topic Editors
Setsuko Komatsu, Hans-Peter Mock, Pingfang Yang, and Birte Svensson

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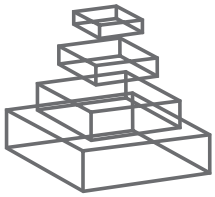

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APPLICATION OF PROTEOMICS FOR IMPROVEMENT IN CROP PROTECTION/ARTIFICIAL REGULATION

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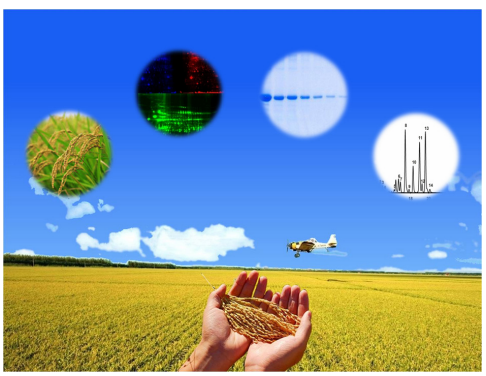

With the availability of genome information for more and more species and the advancement in mass spectrometry technologies, proteomics has come into true since 1990s. As the direct executors for physiological and biochemical reactions, proteins are undoubtedly important in biological system. Proteomics is the subject that focuses on protein entity. How could proteomics contribute in solving different plant biological problems, and how could we apply it in agriculture and plant biotechnology are major issues in plant proteomic studies. Proteomics technology has unraveled a great number of proteins which play crucial roles in plant growth/development and adaptation to environmental stresses. Functional analyses of those proteins will contribute to develop stress-resistant/tolerant crops and artificially regulated crops.

With a rising population, growing more food at affordable prices becomes even more important. On the other hand, he accelerated global warming influences the yield and quality of crop. This challenge requires integrated approaches to increase agricultural production and cope with environmental challenges. Proteomics can play specific roles in addressing the growing demand for food. The advent of proteomics has allowed researchers to identify a broad spectrum of proteins in living systems. This could help to find the functional important proteins or their modifications that could not be found through the studies at any other levels. This capability is especially useful for agriculture because it may give clues not only about nutritional value but also about traits such as yield and how these factors are affected by adverse environmental conditions.

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Application of proteomics for improving crop protection/artificial regulation

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CURRENT STATUS AND CHALLENGES IN CROP PROTEOMICS

The application of proteomics for analyses of crop plants has rapidly increased within the last decade. Although proteomic techniques are routinely used in plant laboratories worldwide, and constitute powerful study tools, there is still considerable room for improvement. In particular, the fraction of the plant proteome that can be detected using current approaches is markedly lower than that of other "Omics" techniques and therefore does not give a complete representation of the cellular proteins.

In the majority of cited papers, two-dimensional electrophoresis (2-DE) gel is the predominant technique used for separating proteins. However, liquid chromatography (LC)-based proteome analysis is becoming increasingly common in many laboratories. Both protein separation techniques have specific advantages. With a standard 2-DE approach, protein modification and degradation can be rapidly visualized, whereas LC-based methods require much lower amounts of starting material. The application of crop proteomics has been hindered by the limited availability of genomic information. However, with the successful development of "next-generation" sequencing technologies, identification and annotation of proteins and their isoforms in a particular crop species is becoming much more straightforward.

A specific advantage of proteomics over other "Omics" techniques is the capacity to reveal post-translational modifications (PTMs), which is a prerequisite to determine the functional impact of protein modification on crop plant productivity. To date, about 300 PTMs have been identified through proteomic analyses. However, major efforts are needed to establish reliable tools and strategies for evaluating the impact of this increasing number of different PTMs in crops. Finally, crop proteomics is expected to become an essential part of integrated "Omics" approaches. However, a major challenge for crop proteomics will be keeping pace with the throughput capacity of other "Omics" techniques. The application of proteomics for the functional analysis of plants will benefit from advances in plant phenotyping. Specifically, improved techniques for the automated, non-invasive phenotyping of plant collections will assist in the selection of appropriate genotypes for proteomics-based functional analyses aimed at characterizing the relevant traits for future crop breeding.

PROGRESS IN CROP PROTEOMICS FOR ARTIFICIAL REGULATION

Proteomic studies have identified numerous proteins that play crucial roles in plant growth and development. However, determining how this wealth of information can be applied toward agriculture and the artificial regulation of crops is a major challenge. Seeds are one of the most important factors in crop production, as seed viability is related to crop yields. He and Yang (2013) applied proteomics to the study of the regulation of rice seed germination and showed that starch is degraded in endosperm and later biosynthesized in the embryo during germination, a process that appears to promote the gradual utilization of nutritional reserves.

Heterosis has been widely used in crop production, in which a sterile male line is critical for hybrid breeding. Identifying the proteins involved in the regulation of male sterility represents a major target in crop proteomic studies (Wang et al., 2013). In contrast to traditional breeding methods, the application of transgenic techniques is becoming increasingly popular to rapidly obtain crops with desired qualities. Evaluation of these genetically modified crops with proteomic methods is essential (Gong and Wang, 2013).

Due to impending changes in the global climate and continued industrialization, maintaining food safety represents a serious challenge worldwide. To sustainably feed the world population, effective methods to increase the efficiency of sunlight conversion are needed (Driever and Kromdijk, 2013). C4 plants are more efficient at light conversion than C3 plants because they contain two different types of chloroplasts. Comparative proteomic analyses of C4 chloroplasts (Zhao et al., 2013) might help to determine the key components that influence the efficiency of sunlight conversion (Manandhar-Shrestha et al., 2013).

The interaction between crops and other organisms is an important factor that influences the growth and eventual yield of crops. For example, the pathogen *Fusarium graminearum* causes head blight of small grain cereals and dramatically reduces grain yield and quality, which has great economic impact on the cereal industry. Proteomic analysis is expected to complement traditional molecular genetics approaches for studying the mechanisms by which this pathogen attacks cereal crops (Yang et al., 2013). The use of proteomics for analyzing the interaction between crops and bacteria, particularly the symbiotic

interactions in legume root nodules (Salavati et al., 2013), has also been demonstrated (Afroz et al., 2013).

Most studies have been conducted on whole organs or tissues, which do not allow for the collection of spatial information. It is therefore expected that the use of MS imaging techniques, which have been successfully applied in the field of medicine, will aid in obtaining information on the spatial distribution of metabolites and proteins (Matros and Mock, 2013).

PROGRESS IN CROP PROTEOMICS FOR STRESS RESPONSES

Stress is a key limiting factor that impairs the growth and yield of agricultural crops. Stressful conditions often lead to delayed seed germination, reduced plant growth, and decreased crop yield. Proteins associated with the primary function of an organ are specifically accumulated in that organ/tissue or organelle. Komatsu and Hossain (2013) highlighted the need for organ-specific proteomic analyses to identify proteins that are commonly accumulated in organs under a wide range of abiotic stresses (Komatsu and Hossain, 2013). Furthermore, due to the nature of abiotic stress, intracellular compartments play a dominant role in plant stress responses. Nouri and Komatsu (2013) reported that a number of subcellularly localized proteins, including ion/water transporters, reactive oxygen species scavengers, and proteins related to signaling and transcriptional regulation, are involved in stress tolerance.

Jacoby et al. (2013) described the application of the emerging proteomic technology of multiplexed selective-reaction monitoring MS, which has increased accuracy and throughput, for enhancing these approaches and providing a clear method to rank the relative importance of the growing cohort of stress-responsive proteins. In addition to crops, proteomic techniques have been applied to the study of moss species that serve as model systems in plant science (Wang et al., 2013) and several agriculturally important fruits (Chan, 2013) under abiotic and biotic stresses. It was revealed that proteins involved in different metabolic pathways in fruit were activated after postharvest treatments, suggesting that biologists should focus on combination treatments to reduce postharvest decay for minimizing production losses (Chan, 2013). For moss, comparison of the stress responses between different treatments revealed that there is a closer relationship between abscisic acid (ABA) and salt or dehydration than there is between ABA and cold (Wang et al., 2012).

Abiotic stresses are major constraints facing global crop production. For example, high temperature impedes the development and growth of crops. Proteomic studies have revealed that activation of amylolytic enzymes by high temperature is a crucial trigger for grain chalkiness (Mitsui et al., 2013). Takahashi et al. (2013) examined responses to freezing stress, which causes serious problems for agricultural management, and found that the plasma membrane plays significant roles in signal perception and cellular homeostasis, indicating that plasma membrane proteins are the most important factors in determining the environmental stress tolerance of plants. Salt stress severely decreases crop production and growth; however, certain crop cultivars show significant tolerance against the negative effects of salinity. Many salt-responsive proteins have been detected in major crops and are thought to increase resistance to salt stress (Aghaei and Komatsu, 2013). Hossain and Komatsu (2013) described the recent contributions of proteomic studies toward the understanding of heavy metal stress responses in plants, particularly the use of redox proteomic approaches for studying heavy metal-induced protein oxidation. The findings presented in the above review article may shed light on the cross talk that appears to occur between different stress signal pathways. The application of proteomic approaches to collect such data will aid in the design of genetically engineered stress-tolerant crop plants.

OUTLOOK

With impending climate changes, a rapidly growing global population that is predicted to exceed 9 billion people within three decades, and increasing need for natural resources, such as water and minerals, greater insights into the foundations of sustainable food production are needed to ensure efficient crop yields and applications. To achieve these objectives, novel tools for protecting crops against biotic and abiotic stresses and for unraveling the mechanisms underlying the development and vitality of seeds are required. "Omics" technologies continue to be promising tools for such explorations. As complete genomes are available for an increasing number of crop and model plants, systems biology or integrated "Omics" approaches will help to unravel the underlying mechanisms of complex plant traits, such as resistance to stresses, at a molecular level. The widespread application of quantitative proteomic techniques in combination with sophisticated imaging techniques for the identification and mapping of PTMs is expected to provide detailed understanding of protein regulation in complex biological networks. Such multidisciplinary strategies will also aid in the design of approaches for mitigating the damaging effects of plant stressors and promoting beneficial plant–microbe interactions. Systems biology analysis will also help in the breeding of robust crop plants that are tolerant to environmental stresses and have high nutritional value. Future crop proteomic studies aimed at understanding the structural basis for the interactions between biological molecules will be critical for controlling the regulation and function of both crop and associated microbial proteins.

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Proteomics of rice seed germination

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Seed is a condensed form of plant. Under suitable environmental conditions, it can resume the metabolic activity from physiological quiescent status, and mobilize the reserves, biosynthesize new proteins, regenerate organelles, and cell membrane, eventually protrude the radicle and enter into seedling establishment. So far, how these activities are regulated in a coordinated and sequential manner is largely unknown. With the availability of more and more genome sequence information and the development of mass spectrometry (MS) technology, proteomics has been widely applied in analyzing the mechanisms of different biological processes, and proved to be very powerful. Regulation of rice seed germination is critical for rice cultivation. In recent years, a lot of proteomic studies have been conducted in exploring the gene expression regulation, reserves mobilization and metabolisms reactivation, which brings us new insights on the mechanisms of metabolism regulation during this process. Nevertheless, it also invokes a lot of questions. In this mini-review, we summarized the progress in the proteomic studies of rice seed germination. The current challenges and future perspectives were also discussed, which might be helpful for the following studies.

Keywords: proteomics, seed germination, rice, metabolism, post-translational modification

INTRODUCTION

For many plants, seed is essential to reproduce and disperse their progenies. It is also an adaptive strategy for plant survival under stresses. Seed germination, which is an early and crucial stage in plant life cycle, refers to the physiological process starting from the uptake of water by the dry seed and ending with the radicle protrusion (Bewley, 1997). The special water-uptake curve depends on the species and genotype. Seed germination is a complex physiological and biochemical process that involves a series of signal transduction and gene expression regulation. As a major branch of the modern seed biology, studies on seed germination have obtained great progresses in the last two decades. In spite of this, how those complex activities are regulated in a coordinated and sequential manner during germination is largely unknown.

Rice is a monocotyledonous model plant and an important food crop. Rice seed germination could determine its seedling growth and yield to some extent. Plants have evolved fine mechanisms (protect and repair from damage) to ensure the seeds to preserve germination capability. Among them, seasonal dormancy is a common selection for environment tolerance (Footitt et al., 2011). However, most cultivated rice seeds have no or shallow degree of dormancy. In some cultivars, the mature seed could germinate in panicles (named as pre-harvest sprouting or vivipary) encountering suitable climate conditions, which reduces seed yield and quality. Genes related to ABA synthesis has been proved to be involved in the pre-harvest sprouting of rice seed (Fang and Chu, 2008). Interestingly, germination is reversible in some species. The late maturation process of seeds can be re-induced partially at the early stages of germination (Lopez-Molina et al., 2002), which might provide an idea for vivipary preventing. Seed storage is often accompanied with a progressive seed aging and loss of germination vigor even under the "best" storage conditions. Rice seed longevity has been proved to be controlled by several genetic factors (Miura et al., 2002). These problems are the main objectives for the studies on seed germination.

Rice is the first crop with the genome being sequenced (Goff et al., 2002; Yu et al., 2002). The whole genome functional annotation and micro-array analyses development make it an ideal monocotyledonous model system in modern plant biological studies. Depending on the expansion of sequence data, several large-scale-omics including transcriptomic, proteomic, and metabolomic methods were established to investigate the mechanisms of seed germination (Holdsworth et al., 2008). Usually, the abundant storage mRNA in the dry seeds can satisfy the protein *de novo* and instant biosynthesis upon imbibition. Although *de novo* mRNA synthesis can help to ensure the rapidity and uniformity of germination, inhibition of transcription by α-amanitin or actinomycin D could not prevent germination; whereas, blocking the translation with cycloheximide resulted in total inhibition of the germination (Rajjou et al., 2004; He et al., 2011a; Sano et al., 2012). Since the entire complex reactions of seed germination are mainly enforced by different proteins, protein profile analysis might be more precise to clarify this physiological process.

With huge available data of genome information and the development of mass spectrometry (MS) technology, proteomics is exerting great influence in analyzing the dynamic and diverse biological processes. A series of comprehensive reviews have summarized the progresses of proteomics and its impacts on rice (Komatsu et al., 2003; Rakwal and Agrawal, 2003; Agrawal and Rakwal, 2006; Agrawal et al., 2006, 2009; Komatsu and Yano, 2006; Agrawal et al., 2011). A literature survey indicates that the

number of proteomic studies on rice seed germination was gradually increased during the early period (1991–2001) and sharply risen in the last decade (2002-; **Figure 1**). However, there is no review on the proteomics of rice seed germination until now. Comparing with another model plant *Arabidopsis*, rice seems to have much more to be elucidated. Here, we review the progresses in the proteomic study of rice seed germination. Meanwhile, the challenges and future perspectives in this field are also discussed.

PHYSIOLOGICAL FEATURES OF RICE SEED IN GERMINATION

Rice seed has a dominant endosperm for nutrient-storage. The starchy endosperm is surrounded by aleurone layer and neighbored with embryo. Between endosperm and embryo, there is scutellum, a metamorphosis of cotyledon. Embryo and endosperm play different roles in rice seed germination. The embryo contains most of the genetic information that control the germination. Upon imbibition, the substrate and energy starvation will activate the embryo to produce phytohormone [mainly gibberellic acid (GA)]. The GAs can diffuse to aleurone and initiate a signaling cascade that leads to synthesis of α-amylases and other hydrolytic enzymes. These enzymes will then secrete into the endosperm to drive the degradation of storage compounds including starch, lipid and protein for seedling establishment (Jacobsen et al., 1995; Bethke et al., 1997; **Figure 2**).

During seed germination, the increasing of total water content or fresh weight follows a classic triphasic model (Bewley, 1997). When germinating in the distilled water, the rice seed weights increased rapidly during the first 20 h imbibition (phase I), and there is no significant morphology changes. The phase I is followed by a stable plateau stage until 50 h (phase II) during which the coleoptiles elongation could be observed at this stage. Phase III is another rapid water uptake stage accompanying with the protrusion of the radical (Yang et al., 2007; **Figure 3**). Phase II was usually regarded as the most important stage, because all of the germination required metabolic reactions are reactivated during this period. However, transcriptome of germinating rice seed indicated that the switch may happen even earlier, since a greater proportion of immediate transcript has been identified at this point (Howell et al., 2009).

A series of exogenous and endogenous factors participate in germination regulation, including water, temperature, light, circadian rhythm, and phytohormones (Penfield et al., 2005; Holdsworth et al., 2008). Water is one of the decisive factors, and germination could be arrested under conditions of water deficit (Hundertmark et al., 2011). Rice is one of the few plants that can germinate anaerobically through a rapid elongation of coleoptile (Menegus et al., 1991; Perata et al., 1997). But the radicle could not protrude well under this condition. Once it is switched to the aerobic condition, the radicle can continue to elongate, which suggested that oxygen availability is another determinant factor for true germination (Howell et al., 2007). For Arabidopsis, pre-cooling the moist seed at low temperature (4°C) would stimulate the germination for water-uptake and dormancy-breaking. However, high temperature sprouting is often used to strengthen rice seed germination. To the contrary, low temperature treatment could injure its radicle.

PROGRESS IN PROTEOMICS OF RICE SEED GERMINATION

Agrawal et al. (2006) have forecasted that rice proteomics is stepping into the second phase that mainly focuses on function analysis and network construction from the first stage characterized by description of proteome profiles. Rice proteomics have also contributed to unveiling the mechanism of the rice seed germination (Table S1; **Figure 3**).

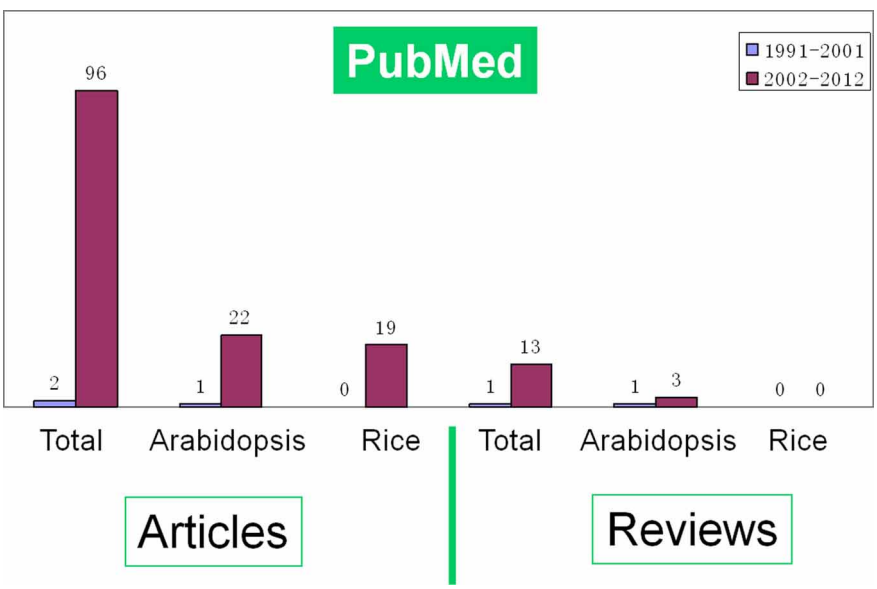

FIGURE 1 | A survey of the literatures indicates the growing number of proteomic research articles and reviews on seed germination in all species (total), *Arabidopsis* and rice respectively during proteome construction period (1991–2001) and sharply rising period (2002-). The data was downloaded from PubMed database.

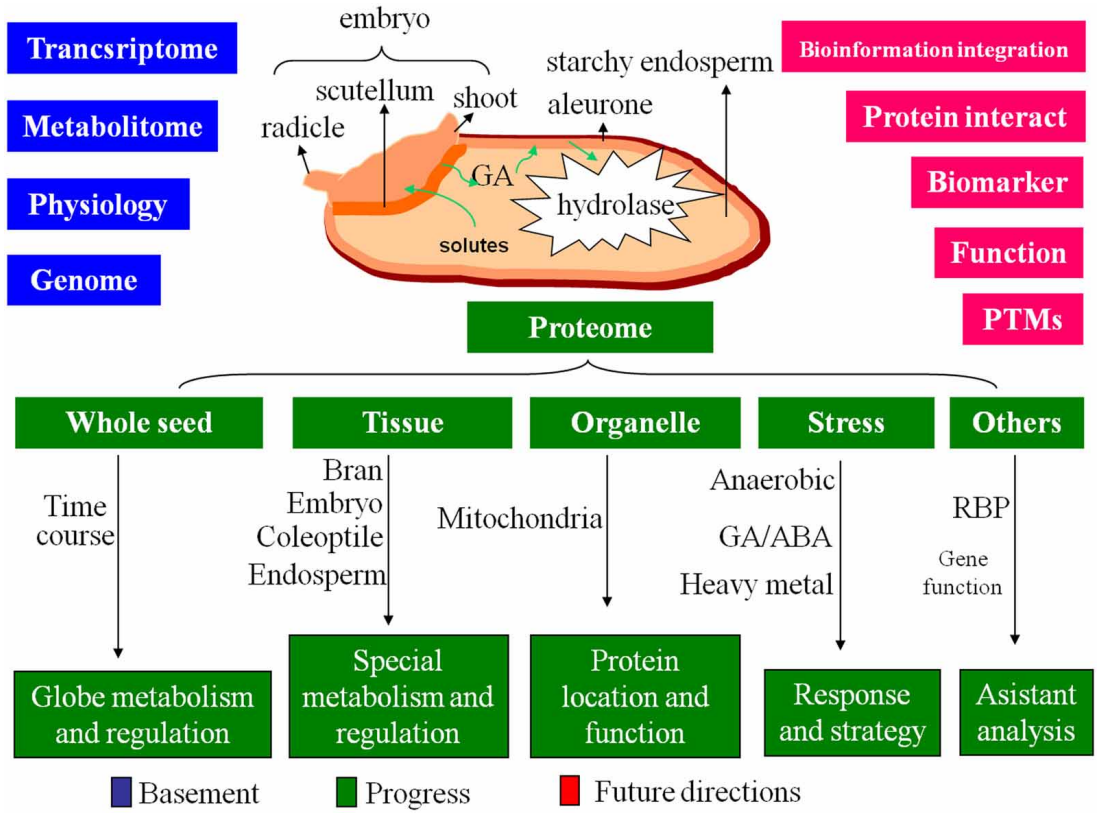

FIGURE 2 | The basement, progress and future directions of the proteome on rice seed germination. Based on the great achievements of the genome, transcriptome, metabolitome and physiology, the proteome of rice seed germination obtained progress on multi-level, future directions will focus on the intensive studies of protein interaction, protein biomarker screening, PTMs, individual protein function analysis, and bioinformation integration.

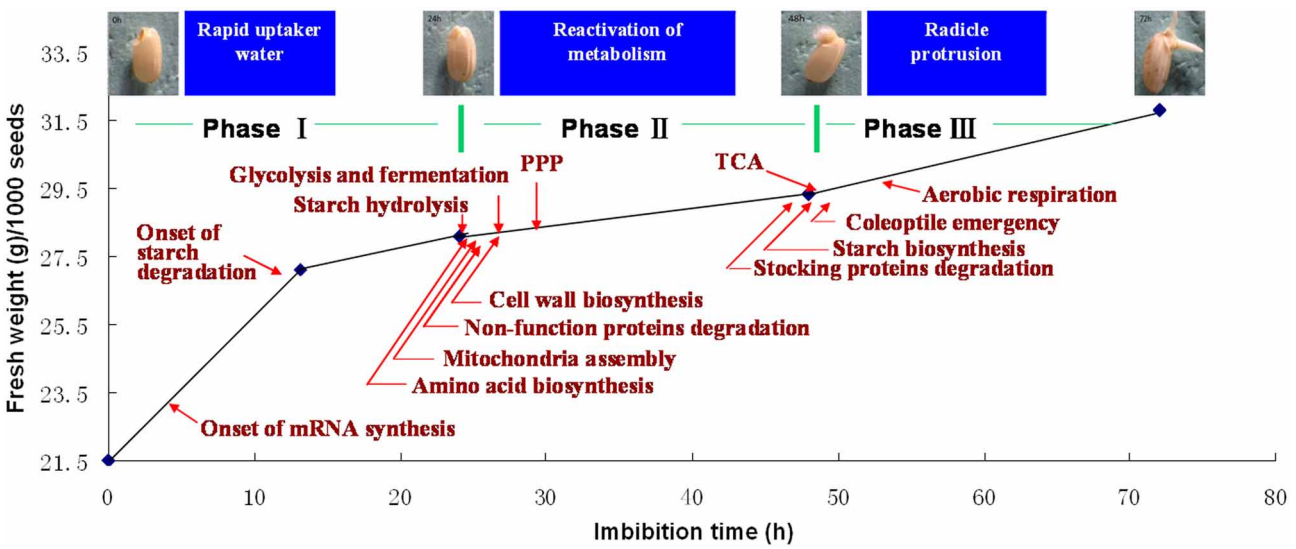

FIGURE 3 | Sequentially happened activities during rice seed germination. Upon imbibition, rice seed germination process could be divided into three phases (Yang et al., 2007). Phase I is the first rapid water-uptake period with the onset of mRNA biosynthesis (Howell et al., 2009), phase II is the most important stage for metabolism reactivation, mobilization of reserves, cell structure repair, cell wall loosening, and coleoptile elongation, phase III is another rapid water-uptake stage with TCA and aerobic respiration recovering, cell division initiation, radical protrusion, and initiation of seedling establishment. The picture displayed the rice seed after 0, 24, 48, and 72 h imbibition (provided by Mr. Chao Han).

To give an overall view of the protein mobilization during the rice seed germination, comparative two-dimensional electrophoresis (2-DE) of whole germinating seeds with a time-course sampling were carried out on different rice species (*Oryza sativa* cv. Indica. 9311 and *Oryza sativa* cv. Nipponbare) by Yang and his coworkers (Yang et al., 2007; He et al., 2011b), 148 and 39 proteins were displayed differentially in the process, respectively. The drastic changed patterns of these proteins indicate that germination not only consumes reserves, but also bestows the plant with defenses and rebuilds the morphology. Storage proteins and some seed development- and desiccation-associated proteins were down-regulated; conversely, catabolism-associated proteins were up-regulated with the extension of imbibition time. It deserves to be mentioned that the degradation of seed maturation and desiccation associated proteins (24 h imbibition) mainly occurred a little early than that of storage proteins (48 h imbibition, **Figure 3**). Since phase II was the most important stage in seed germination, acquiring a comprehensive knowledge of this stage is necessary. Protein profile was obtained from the germinating rice seeds at 24 h after imbibition through 1-DE via liquid chromatography and tandem MS (LC-MS/MS) proteomic shotgun strategy (He et al., 2011a). Totally, 673 proteins sorted into 14 functional groups were identified. Based on the proteomics data, a comprehensive metabolic and regulatory pathway was constructed, which contributed to understanding the metabolic style, regulation of redox homeostasis, and gene expression during rice seed germination. The results also suggested that mobilization of reserves occured during rice seed germination not only by means of central carbon metabolism pathways (glycolysis and TCA cycle) but also by fermentation pathway (**Figure 3**).

The embryo plays a decisive role during seed germination, so protein profile analysis in the germinating seed embryo may be helpful in unraveling the complex mechanism of this process. Proteins were extracted from 1 or 2 d imbibied embryo of rice seed and analyzed by 2-DE (18 cm IEF tube gel). Sixty differentially expressed proteins were identified and sorted into 10 categories by their functions: metabolism, oxygen-detoxifying, protein processing/degradation, stress/defense, energy and others. This suggested that there are multiple regulations in embryo during seed germination (Kim et al., 2009).

Coleoptile is a protective sheath covering the emerging shoot. For germinating rice seed, the anoxia stress can induce the expression of α-amylase (*RAmy3D*) to degrade the starch and provide substrates for elongating rice seed coleoptiles (Perata et al., 1992). Coleoptile elongation was suppressed in a *cipk15* mutant under submergence and was rescued by adding exogenous sucrose (Lee et al., 2009). Using DIGE and iTRAQ analysis, 140 and 142 differentially expressed proteins were identified from the coleoptiles treated by 4 d of aeration vs. 6 d of anoxia as well as 4 d of aeration with an additional 1 d under anoxia. Proteins related to translation and antioxidant defense were significantly up-regulated in anoxic coleoptiles. Furthermore, accumulation of amino acids (serine, glycine, and alanine), which might be synthesized from glyceraldehyde-3-phosphate or pyruvate, was also detected. This result indicates that amino acid biosynthesis might contribute to anoxia tolerance in cells besides sugar and energy (Shingaki-Wells et al., 2011).

Aleurone is a living tissue surrounding the starchy endosperm and synthesizing key enzymes for germination. The single layer aleurone (unlike multiple-layers in barley) is difficult to be separated in rice seed manually, but the aleurone-rich bran might be selected as a substitute. A complete protein snapshot combined with both gel-based (1-DE and 2-DE) and gel-free (LC-MS/MS) strategies were applied on full-fat or defatted rice bran, 43 unique proteins were identified including signaling/regulation proteins (30%), proteins with enzymatic activity (30%), storage proteins (30%), transfer (5%) and structural (5%) proteins (Ferrari et al., 2009). Yano et al. (2001) had developed a disulfide proteome technique to visualize redox changes in proteins. Rice bran was analyzed by the disulfide proteome technique, and embryo-specific protein 2 (ESP2), dienelactone hydrolase, putative globulin, and globulin-1S-like protein were identified as putative target of thioredoxin, which support the hypothesis that thioredoxin activates cysteine protease with a concurrent unfolding of its substrate during germination (Yano and Masaharu, 2006).

GA and ABA (abscisic acid) are two main phytohormones that antagonistically regulate seed germination by modulating GA- and ABA-responsive functional proteins. GA can promote seed germination and break dormancy, while ABA can induce dormancy at the later phase of seed maturation. GA and ABA pathways might interact to each other by directly inhibiting the synthesis of some proteins or regulating the common target proteins such as DELLA. A proteomic analysis of rice germination under GA and ABA modulation was carried out on embryonic tissue, and 16 notably modulated proteins were identified, including multi-functions: metabolism, oxygen-detoxification, stress/defense functions, protein processing/degradation, signaling and cell wall structure. Western blot and immunolocalization analysis of two differentially expressed protein, rice isoflavone reductase (*OsIFR*) and rice PR10 (*OsPR10*), revealed that both proteins were specifically expressed in the embryo and dramatically down regulated by ABA, which suggested that proteins in the embryo rather than endosperm might be more sensitive to phytohormones (Kim et al., 2008a).

Seed germination provides a good system for studying mitochondria biosynthesis. As an endosymbiotically derived organelle, the typical cristae-rich mitochondria cannot be *de novo* synthesized, but are developed from unstructured double membrane bounded pro-mitochondria. Transcriptome revealed that a greater proportion of transcripts encoding proteins located in the mitochondria peaked early at 1 or 3 h after imbibition (Howell et al., 2009). Coincident with this was the rapid changes in mitochondrial protein content and function. Comparison between embryo mitochondrial protein profiles of dry seed and 48 h imbibed seed, 56 differentially expressed proteins were identified including the outer membrane channel TOM40 and the inner membrane TIM17/22/23 families. Usually, proteins involving import apparatus are very few in the mature mitochondria; the accumulation of these import proteins in the dry seed could operate functions after 2 h imbibition, and then sequentially assemble the components for TCA cycle and electron transport chain (Howell et al., 2006). Anoxic germination also provides a system for oxygen signals studies. Comparison between mitochondrial

protein profiles of rice seed germinated for 48 h at anaerobic and aerobic conditions showed that 13 proteins appeared differently in abundance. Although three proteins from the TIM17/22/23 family were 6–14 folds up-regulated under anaerobic conditions, the capacity of the general import pathway was found to be significantly lower in mitochondria under anaerobic conditions than that under aerobic conditions. In this system, import capacity was ultimately dependent on the presence of oxygen; which provided a link between the respiratory chain and protein import apparatus (Howell et al., 2007).

Dry rice seeds contain more than 17,000 stored mRNAs (Howell et al., 2009). The RNA-binding proteins (RBP) perform important role in keeping the stability and regulating the functions of those long-lived mRNAs. Masaki et al. (2008) fractionated RBP in dry rice seeds by single stranded DNA (ssDNA) affinity column chromatography, and revealed three types of RBP in mature seeds: Khomology (KH) domain containing protein, putative RNA- binding protein and glycine-rich RNA-binding protein. The putative RNA-binding protein and glycine rich RNA-binding protein were down-regulated after seeds imbibition, which might promote germination. It is still unknown how the biosynthesis of different proteins happens sequentially during germination. Comparative proteomic analysis on rice seed embryos dissected at 0, 2 and 4 d treated with transcriptional inhibitor actinomycin D (Act D) revealed 20 up-regulated proteins including some carbohydrate metabolism and cytoskeleton formation related proteins. Among them, the translation timing of 8 proteins was clearly later than that of the other 12 proteins. This indicated that the long-lived mRNAs present in dry rice seed are selectively translated depending on the germination phase (Sano et al., 2012). This selective translation machinery also has been observed in maize seeds germination (Jime'nez and Aguilar, 1984).

Because of their sessile character, plants have to confront with many different adverse environments during their whole life. In nature, plant seeds have to overcome different stresses to ensure a survival. These stresses include salt, dehydration, osmotic, and extreme temperature. Currently, with the rapid industrial development, heavy metals, such as copper, cadmium, and arsenic, are also a serious problem in agricultural production. The molecular mechanisms of resistance to these stresses have been systematically conducted in Arabidopsis seed germination (Daszkowska-Golec, 2011). Such studies were also performed in rice during its seed germination. Under copper stress, the germination rate, shoot elongation, plant biomass, and water content all decreased in rice, whereas TBARS (thiobarbituric acid reactive substance) content accumulated quickly. Proteomic analysis revealed that 18 proteins were up-regulated in germinating rice seed under copper stress, including some antioxidant and stress-related proteins such as glyoxalase I, peroxiredoxin, aldose reductase, which suggested that excess coppers could generate oxidative stress. Key metabolic enzymes such as α-amylase and enolase were down-regulated, which indicated excess copper could affect water uptake and reserve mobilization (Ahsan et al., 2007a). Arsenic stress was also reported to decrease α-amylase activity in germinating wheat seeds (Liu et al., 2005). Cadmium also induced the expression of proteins related to defense, detoxification and antioxidant during rice seed germination (Ahsan et al., 2007b). Some low molecular-weight proteins participate in detoxification of heavy metals. Sixteen proteins in the 6–25-kDa range were identified by embryo proteome of rice seeds germinated in presence or absence of 200 mM Cu for 6 days. This study provided the first proteomic evidence that metallothionein and CYP90D2 were Cu-responsive proteins in plants (Zhang et al., 2009).

Proteome analysis has superiority in obtaining specific evidence on the function of designated gene. Komatsu et al. (2005) had investigated Gα (a subunits of heterotrimeric G proteins) protein-regulated proteins in the rice *dwarf1* (d1) mutant. The mutant showed abnormal morphology with shortened internodes, dark green leaves, and small-round grains. While a constitutively active mutant (QL/d1) of the Gα protein produced large round seeds. Using 2-DE, 7 seed embryo proteins were identified down-regulated in the d1 mutant. A receptor for activated C-kinase (RACK) was discovered decreased in d1 mutant but increased in QL/d1. The RACK would disappear after 24 h imbibition, while ABA could promote its expression, which suggested ABA could promotes the expression of both Gα protein and RACK that controls rice seed germination. These data showed that RACK was also regulated by Gα-protein and played an important role in rice germination.

Biomarker hunting is an important purpose for proteomic study; and proteomics is proved to be a powerful tool in screening the biomarker. Probenazole-inducible gene (PBZ1) is a protein marker that was successfully identified by proteomic analysis of rice lesion mimic mutant (spl1) for cell death (Kim et al., 2008b). Phytic acid can form stable complexes with divalent cations and decrease its bioavailability. Comparative proteome analysis of dry seed was performed between the low phytic acid (lpa) rice line (Os-lpa-XS110-1) and its parental line (Xiushui 110, XS-110). Two major differentially expressed proteins, triose phosphate isomerase (TPI) and fructose biphosphatealdolase (FBA) were found to be involved in phytic acid metabolism. These two proteins might be developed as biomarkers for rice seed quality evaluation (Emami et al., 2010). Based on our study (Yang et al., 2007), a starch degradation enzyme α-amylase was sharply increased during rice seed germination. This protein might be an ideal candidate biomarker for rice seed germination.

Seed hereditary is the main factor influencing germination. Hybrid rice cultivars usually have high germination vigor. Comparison of the proteome profiles of mature rice embryo were conducted in hybrid cultivar and its parental lines. Most of the storage proteins exhibited over-dominance, and stress-induced proteins displayed additive effects, which might contribute to abiotic or biotic stress resistance (Ge et al., 2008). In addition to starch, rice endosperm also reserves lots of proteins. Comparative proteomic analysis of endosperm proteins was carried out on two hybrid *indica* cultivars [Liangyoupeijiu (LYP9) and Shanyou63]. Although obviously differences existed in morphology, physiology, and grain quality, the 2-DE profiles displayed that they had nearly the same protein compositions, except for some isoforms of peroxiredoxin, aldose reductase and granule-bound starch synthase were specific in LYP9 or Shanyou63 (Yang et al., 2006).

PROTEINS INVOLVED IN RICE SEED GERMINATION

Upon imbibition, vivid rice seeds should reboot the system activity and mobilize the reserves for germination. Since protein is the real executor of life activities, a series of proteins will participate in the germination process. Some indispensable proteins for germination will be discussed here, such as those related to metabolic, storage, protein synthesis, ROS scavenger, signaling to name a lot.

Without exception, metabolism-related proteins, especially those involved in the carbohydrate metabolic pathways including major and minor carbohydrates metabolism, glycolysis, TCA cycle, fermentation, gluconeogenesis and glyoxylate cycle and pentose phosphate pathway (PPP), were the major group of proteins existing in germinating rice seed (**Figure 3**). Since starch is the major reserve of the rice endosperm, all the enzymes involved in starch degradation to hexose phosphate were identified in rice seeds at 24 h of germination. Except for α- amylase that was greatly increased in abundance at the late stage of phase II, all the other enzymes keep constant in the whole germination process (Yang et al., 2007; He et al., 2011a), which suggested that the starch degradation metabolism is vigorous at the phase II. The hexose phosphate from the degradation of starch will further experience glycolysis. Totally, 22 enzymes that catalyze all the steps in the glycolysis pathway were detected, and most of them were up-regulated at 12 h imbibition. Pyruvate, the final product of glycolysis, could be further degraded through TCA cycle; most TCA cycle-related proteins were also identified as up-regulated proteins (Yang et al., 2007). Kim et al. (2009) had detected that both succinyl-CoA ligase and cytoplasmic malate dehydrogenase were stably accumulated during germination. TCA cycle might, along with glycolysis, provide the main energy at the late stage of germination. Due to lack of functional mitochondria, the aerobic respiration was detected very low during the first 48 h of imbibition. Anaerobic respiration pathway, such as fermentation, might be the main source of energy at the early stage of germination, which is supported by the identification of lactate dehydrogenase (LDH), pyruvate decarboxylase and alcohol dehydrogenase during this period (He et al., 2011a,b; Yang et al., 2007). Most of the enzymes involved in the PPP also existed in the germinating seeds, and the glycolytic enzymes were identified to be carbonylated and degraded during germination (Job et al., 2005). Blocking glycolysis and reorienting the glucose flux to the PPP could provide cells not only with reducing power in the form of NADPH to surmount oxidative stress, but also pentose phosphate for nucleotide metabolism (Arc et al., 2011).

To be mentioned, all the enzymes related to starch biosynthesis also exist in the embryo of germinating rice seeds. The accumulation of starch granules had been observed in rice embryos during germination, which suggested the solute from the endosperm can be re-synthesized to starch at the embryo (Matsukura et al., 2000; He et al., 2011a,b). But how those solutes were transferred from endosperm to embryo is not understood well. To reboot the quiescent system, *de novo* synthesis of new functional proteins is necessary. Enzymes that related to protein biosynthesis, modification, targeting, and folding, including ribosomal proteins, translation initiation or elongation factors, amino acid activation proteins, trafficking, and secretion proteins and chaperonins, were identified during germination. On the contrary, the storage proteins, seed maturation associated proteins, embryogenesis proteins and proteins related to desiccation were degraded to provide primary amino acids and reduced nitrogen for the seed germination. Although the lipids are not the major reserves, the storage lipids did accumulate during the grain filling and stored in aleurone cells of the cereal seeds (Krishnan and Dayanandan, 2003). β-oxidation and glyoxylate cycle are the two major fatty acids degradation pathways. Enzymes involved in these two pathways were detected during rice seed germination. Interestingly, fatty acid biosynthesis related enzymes such as ATP: citrate lyase and some transacylase had also been detected. Most of amino acids biosynthesis and degradation related proteins also had been identified during rice seed germination. Since most of the biosynthesis and degradation metabolisms are in concurrence, we may wonder when storage reserves should be mobilized during seed germination. Gallardo et al. (2001) inferred that the potential mobilization might exist not only in germination but also in the maturation phase based on the fact that both precursor forms and proteolyzed forms of the 12S seed storage-protein subunit were identified in dry mature seeds.

Reactive oxygen species (ROS) are produced in all living organisms. The ROS might be bifunctional, and over accumulation of ROS can result in oxidative stress. Reducing the oxidized proteins is another critical way to cope with ROS. Upon imbibition, the contents of ROS were gradually increased. ROS can be efficiently scavenged by the antioxidant enzymes like superoxide dismutases (SODs), glutathione S-transferase (GST), catalase, peroxidases, and enzymes in the ascorbate–glutathione cycle. Many of these redox regulation proteins were identified during germination (He et al., 2011a). Carbonylation, one of the important post-translational modifications (PTMs) under oxidative stress (Nystrom, 2005), can not only provoke the degradation of reserve proteins, but also be fatal by targeting those physiological important proteins (Job et al., 2005). Meanwhile, accumulation of NO upon seed imbibition can help to regulate the redox homeostasis by S-nitrosylation of the critical protein thiols and protect them from oxidation (Lounifi et al., 2013). So keeping the redox homeostasis is necessary *in vivo*.

Stress responsive proteins were accumulated during the seed maturation in order to survive from dessication. Among them, HSPs were the largest group. Proteins including universal stress proteins (USP), dehydrins, DNA J family, and late embryogenesis abundant (LEA) proteins can also be regarded as desiccation stress responsive proteins (Yang et al., 2007; He et al., 2011b). The metabolism of nucleotides was not active at early stage of germination since few enzymes required for nucleotide metabolism were detected. However, all 8 except proteins for polygalacturonase proteins involved in the cell wall biosynthesis were identified at 24 h after imbibition, which implied cell wall biosynthesis is active during the phase II (He et al., 2011b; Sano et al., 2012).

CHALLENGES AND PERSPECTIVES

In the last two decades, great progresses have been made in rice proteomics, which provide a comprehensive snapshot on the understanding of rice development, stress tolerance, organelle, secretome, protein post translational modification (PTM), transgenic plant screening and food-safety evaluation

(Agrawal et al., 2011). Among them, proteomics of rice seed germination has also been widely conducted. However, proteomics of rice seed germination is still at its initial stage, and there are still many challenges in this field. Overcoming these challenges might be able to drive the study forward.

The first one is a common challenge for the proteomic study in plant. It is the establishment of routine and reliable method for sample preparation, including tissue harvesting and protein extraction. Previously, mixed tissues were always used to substitute the specific tissue because of the limitation in different sample separation techniques, such as using bran to imitate aleurone (Ferrari et al., 2009). Since different tissues function differently during germination, it is necessary to obtain pure tissues in order to deeply understand the mechanisms. In the future, some specific tiny samples such as aleurone and scutellum might be directly harvested with laser microdissection technique (Nakazono et al., 2003). As for protein extraction, it is critical to extract maximum number of proteins with high quality. Unfortunately, because of the existence of very high abundant storage proteins and basic proteins; it is almost impossible to obtain the protein sample with full coverage (Lee et al., 2008). We would like to suggest the combination of different techniques in protein extraction.

Secondly, protein's function is correlated to its cellular location and PTM, so sub-proteomics study of different organelles or modifications might be very helpful in understanding the mechanisms of seed germination. Up-to-date, very few organelle or modified proteomics studies have been conducted in rice seed germination because of the difficulties in isolation and enrichment of subcellular compartments (Agrawal et al., 2011). Only mitochondrion proteomic studies have been reported in germinating rice seed (Howell et al., 2006, 2007). Comprehensive subcellular proteomes covering more organelles (i.e., nuclear, ER, ribosomes, and protein storage vacuole) should be implemented in combination with optimized method and high-throughput technology. FFE (Free-flow electrophoresis, Zischka et al., 2006) might be a good choice for separating different organelles on the basis of their surface charge. The PTMs can affect protein localization, complex formation, stability, and activity (Ytterberg and Jensen, 2010), and play very important roles in dormancy release and metabolism resumption. More than 300 potential PTMs *in vivo* (http://www.abrf.org/index.cfm/dm.home) have been identified, such as phosphorylation (protein function reactived related), ubiquitination (storge protein degredation related), carbonylated (reactive oxygen related) and S-nitrosylation (nitrogen species related) (Arc et al., 2011). Studies on these protein modifications will undoubtedly contribute a lot in exploring the mechanism of rice seed germination in the future.

Thirdly, huge amount of data on rice proteome have been generated in the last 10 years. However, it is still a challenge to integrate these increasing data and provide a comprehensive insight into the germination process. Comparing with those database for Arabidopsis and soybean (Agrawal et al., 2011; Ohyanagi et al., 2012), few proteome databases had been constructed on rice (Komatsu, 2005; Helmy et al., 2011). However, the database has not been updated in time. In addition, various "omic" data can also provide additive information. So it is necessary to integrate proteomic data with the available genomic, transcriptomic, and metabolomic data in order to comprehensively understand the process of germination.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Proteomics/10.3389/fpls.2013.00246/abstract

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Gene, protein, and network of male sterility in rice

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Rice is one of the most important model crop plants whose heterosis has been well-exploited in commercial hybrid seed production via a variety of types of male-sterile lines. Hybrid rice cultivation area is steadily expanding around the world, especially in Southern Asia. Characterization of genes and proteins related to male sterility aims to understand how and why the male sterility occurs, and which proteins are the key players for microspores abortion. Recently, a series of genes and proteins related to cytoplasmic male sterility (CMS), photoperiod-sensitive male sterility, self-incompatibility, and other types of microspores deterioration have been characterized through genetics or proteomics. Especially the latter, offers us a powerful and high throughput approach to discern the novel proteins involving in male-sterile pathways which may help us to breed artificial male-sterile system. This represents an alternative tool to meet the critical challenge of further development of hybrid rice. In this paper, we reviewed the recent developments in our understanding of male sterility in rice hybrid production across gene, protein, and integrated network levels, and also, present a perspective on the engineering of male-sterile lines for hybrid rice production.

Keywords: gene, male sterility, molecular mechanism, protein, rice

As one of the highest yield crops, rice (*Oryza sativa*) has been used as a staple food for people over 10,000 years. Being high in carbohydrates, low in fat, and rich in protein, rice has become one of the major food supplies for the expanding world population. Today, over 50% of the global population and three quarters of the Asian population live on rice. However, facing the challenge of population explosion and reducing amount of cropland in the world, it is obvious that improve the yield of cereal crops like rice, wheat, and corn are the only way to solve this problem (Lin and Yuan, 1980; Stuber, 1994). Utilizing heterosis is an effective approach to increase crop plant grain yields, and hybrid rice has been popularized in China over the last 30 years. Hybrid rice is mainly based on the three-line and two-line male-sterile systems. Three-line male-sterile system is comprised by the male-sterile line, maintainer line and restorer line, defective nuclear-cytoplasm interaction in the male-sterile line leads to sterility. Boro II (BT), Honglian (HL), and Wild abortive (WA) are three major types of cytoplasmic male sterility (CMS) resources, and are used for the breeding of three-line hybrid rice (Lin and Yuan, 1980). The two-line hybrid rice system is based on the discovery and application of environmentally sensitive genetic male-sterile lines (EGMS), which serve as both the male-sterile lines and maintainer lines under different environmental conditions. Photoperiod-sensitive (PGMS) lines and thermo-sensitive (TGMS) lines are two major types of EGMS germplasm resources. During the past two decades, great achievements have been made in improving rice yields in China using two-line hybrid rice (Yuan, 1994). Since hybrid rice is a major approach for increasing the yield potential of rice, understanding the molecular mechanisms underlying these male sterility lines in rice is critical for improvements in rice hybrid seed production technology. Recently, genes responsible for the CMS and EGMS trait in rice have been cloned. This article focuses on recent investigations of genes and proteins that are associated with male sterility in rice and provides novel insights into male-sterile pathways of rice.

CMS IN RICE

CMS is a maternally inherited phenomenon found in the plant kingdom (Young and Hanson, 1987). CMS leads to pollen abortion but does not affect female fertility and vegetative growth, and fertility can be rescued by fertility restorers (Rf). The CMS/Rf system eliminates the need for hand emasculation; therefore, it is important in commercial hybrid seed production. In most cases, the failure of pollen development in a CMS background is suggested to be associated with chimeric open reading frames (ORFs) that have arisen from unusual recombination events in mitochondria (Linke and Borner, 2005). Many mitochondrial genes that responsible for CMS can be suppressed or counteracted by the products of one or more nuclear genes known as *restorer-of-fertility* genes (Hanson and Bentolila, 2004). Thus, apart from the agronomic importance of CMS, study of the trait of CMS provides a convenient way to dissect genetic interactions between the mitochondria and the nucleus in plants. However, the pollen-specific phenotype in CMS system, and the technical infeasibility of isolating and studying mitochondria from this tissue, increases the difficulties in studies the mechanism of CMS in plants.

In rice, there are three CMS/RF systems, which are named CMS-BT, CMS-WA, and CMS-HL. They have been defined by distinct genetic and cytological features.

CMS-BT

The BT-type rice is the most fully characterized system of CMS in rice. In BT-type CMS, the cytoplasm derived from the rice line Chinsurah BoroII causes male sterility when combined with the nucleus from the rice line Taichung 65 that carries no restorer gene (Shinjyo, 1969). In the mitochondrial genome of Chinsurah Boro II, a chimeric gene called *orf79*, located downstream of e *atp6*, which encodes a cytotoxic peptide, was confirmed to be responsible for the gametophytic male sterility of CMS-BT rice by transgenic experiments (Iwabuchi et al., 1993; Akagi et al., 1994; Wang et al., 2006b). Fertility restoration of CMS-BT rice is controlled by a nuclear gene, *Rf1*, which encodes a PPR-containing protein (Kazama and Toriyama, 2003; Akagi et al., 2004; Komori et al., 2004). Wang et al. reported that *Rf1* locus consists of two *Rf* genes, *Rf1a,* and *Rf1b* (Wang et al., 2006b). Both of the two *Rf1* genes can restore CMS-BT rice by silencing *orf79* transcript via different mechanisms respectively, endonucleolytic cleavage for RF1A and degradation of the dicistronic RNA for RF1B. However, when the two restorers are both present, the *Rf1a* gene has an epistatic effect over the *Rf1b* gene in the mRNA processing (Wang et al., 2006b). It has also been reported that the processed co-transcript of *B-atp6-orf79* is partially degraded, and the unprocessed *B-atp6-orf79* RNA possess the capability to translate. Thus, a certain amount of ORFH79 protein did accumulated in transgenic rice, however, the transgenic line did display a fertile phenotype (Kazama et al., 2008). This suggests a certain level of accumulation of ORF79 in rice does not result in CMS. The quantitative analysis of the amount of ORF79 protein that needs to accumulate to lead to pollen abortion remains to be researched.

CMS-WA

Wild-Abortive CMS (WA-CMS) system is derived from the common wild species *Oryza rufipogon* Griff, which is applied most often for hybrid rice production (Lin and Yuan, 1980). This line shows a distinct difference both genetically and cytologically from that of CMS-BT and CMS-HL. Pollen abortion in CMS-WA occurs relatively earlier during microspore development, mainly at the uninucleate stage. It is sporophytically male-sterile, and the aborted pollen are amorphous. In contrast, the male sterility in both CMS-BT and CMS-HL rice is of the gametophytic type. The molecular mechanism of CMS-WA is poorly understood compared with that of CMS-HL and CMS-BT. Previously, an unedited 1.1 kb mitochondrial *orfB* gene transcript was found to be the candidate sterility gene of WA-CMS by RFLP (Das et al., 2010). But recently, Bentolila and Stefanov suggest *orf126* is the CMS-related candidate gene based on comparison of mitochondrial genomes by next-gen sequencing (Bentolila and Stefanov, 2012). The CMS and Rf genes of CMS-WA have still not been finally identified and cloned.

CMS-HL

The CMS-HL line of rice was developed by the repeated backcrossing of a red-awned wild rice (*Oryza. rufipogon*) from Hainan Island, China with an early maturing indica variety called Lian-Tang-Zao. Hybrid rice varieties based on CMS-HL have been widely grown in China since the beginning of this century because it performs better agronomically and produces higher quality grain than those varieties based on other CMS systems (Liu et al., 2004). Compared with CMS-BT and CMS-WA, more systematic research has been undertaken to investigate CMS-HL rice. Wan and Li reported accumulation of high levels of ROS, significantly decreased adenylate content, as well as ATP/ADP ratio, and reduced mitochondrial membrane potential in Yuetai A compared with Yuetai B during microsporogenesis (Li et al., 2004; Wan et al., 2007). A similar phenomenon was also reported in other CMS systems such as *Brassica napus* and *Nicotiana tabacum* (Bergman et al., 2000; Teixeira et al., 2005). In fact, lower ATP levels were also found in their vegetative development, but only the pollen function is lost, suggesting that the mitochondrial activity is strictly required for gametophyte development during microsporogenesis in these lines.

In CMS-HL rice, a chimeric mitochondrial gene called *orfH79*, located downstream of *atp6*, has been proposed as the candidate gene causing the CMS trait of Honglian rice (Yi et al., 2002). Peng et al. confirmed the expression of *orfH79* result in the pollen abortion in HL-maintainer line using transgenic experiments (Peng et al., 2010). The nuclear Rf5 gene can restore the fertility of CMS-HL rice, moreover, a Gly-rich protein, GRP 162 were found to interact directly with RF5 to form a subunit of the restoration of fertility complex. This complex processes the CMS-associated transcript *atp6-orfH79*, which provides a new perspective on the molecular mechanism underlying fertility restoration (Hu et al., 2012).

To better understand the molecular mechanism of HL-CMS rice, proteomic analysis has been performed. Two-dimensional gel electrophoresis and isotope-code affinity tag (ICAT) technology have been used to analysis the protein expression profile between the Yuetai A and Yuetai B. Results showed that proteins associate with energy production were reduction in the anther of HL-CMS rice (Wen et al., 2007; Sun et al., 2009), suggesting a low level of energy production played an important role in inducing CMS-HL. Accumulation of ORFH79 in mitochondria impairs their normal function, proteomic analysis showed energy production protein were reduced in anther of CMS-HL rice, and physiological features showed the mitochondrial activity in CMS-HL rice was down-regulated. This inspired study of the mitochondrial complex proteome between Yuetai A and Yuetai B. BN-PAGE and mass spectrometry- based quantitative proteomics showed a reduced quantity of mitochondrial complexes in Yuetai A compared to Yuetai B, indicating a defect in mitochondrial complex assembly in the sterile line. This study gave us a new viewpoint on the mechanisms of CMS (Liu et al., 2012).

A series of CMS-associated mitochondrial genes in rice and other plants have been cloned and functionally studied, but how CMS proteins specifically cause pollen abortion remains unclear. A platform for studying mitochondria of rice reproductive tissue is the main obstacle to this study. More dexterous and sensitive systems need to be developed to study the mechanism of CMS in rice and other plants.

EGMS IN RICE

Nongken 58S (NK58S), is the first spontaneous photoperiod-sensitive genic male-sterile (PGMS) mutant found in the japonica cultivar Nonken 58 (NK58) grown in Hubei Province, China in 1973. Large-scale application of two-line hybrid rice in agriculture was developed quickly with the discovery of NK58S (Shi, 1985; Shi and Deng, 1986). The fertility of NK58S is highly regulated by day length at specific inflorescence development stages. It retains complete male sterility when the day length (photoperiod) is longer than 14 h during the anther development, however, male fertility returns gradually when the day length is shorter than 14 h. In addition, the photoperiod-sensitivity can be affected by temperature: a high temperature slightly promotes complete male sterility under long-day conditions (He and Yuan, 1989). Peiai 64S (PA64S) is one such NK58S-derived line with an indica (*O. sativa* ssp. indica) genetic background. With wide compatibility and good agronomic traits, PA64S has become the most widely used female parent for two-line hybrid rice breeding. However, the fertility transition of PA64S is controlled mainly by temperature rather than by day length: PA64S exhibits male sterility at temperatures higher than 23.5°C during the anther development, but it converts to male fertility when the temperature is ∼21–23°C. Long-day (14 h) conditions can suppress the degree of sterility to fertility conversion under low temperatures (21–23°C), but short-day (12 h) conditions cannot restore male fertility under high temperatures (Luo et al., 1992; Xu et al., 1999; Lu et al., 2007). Although two-line hybrids developed using this EGMS germplasm have made great achievements in improving rice yield in China during the past two decades, people knew less about the molecular mechanism of how the day length and temperature coordinately regulate the fertility transition of EGMS in rice.

Genetic analysis found that *pms3* located on chromosome 12 was the original mutation which converts Nongken 58 to become the PGMS rice NK58S. Recently, *pms3* was cloned and shown to encode a long non-coding RNA (lncRNA) named LDMAR. A sufficient amount of LDMAR is required for male fertility under long-day conditions. A spontaneous G-C mutation causing a SNP between NK58 and NK58S, eventually brings about heritable increased methylation in the promoter region of LDMAR, which reduces the level of LDMAR expression. This then results in premature programmed cell death (PCD) in anther development under long days, and hence male sterility (Ding et al., 2012a). In addition, Ding et al. reported that RNA-dependent DNA methylation (RdDM) is involved in the regulation of PGMS. Promoter siRNA of LDMAR derived from *AK11270* is associated with the DNA methylation level of LDMAR, which reduces the expression level of LDMAR, and therefore male sterility in Nonken 58S under long-day conditions (Ding et al., 2012b).

P/TMS12-1, which confers PGMS in the japonica rice line NK58S and TGMS in the indica rice line PA64S, encodes a unique non-coding RNA, which produces a 21-nucleotide small RNA named *osa-smR5864w*. This RNA shares identity with the product of *pms3* at the nucleotide level, which is responsible for the fertility of the pollen of NK58S and PA64S (Zhou et al., 2012). Taken together, these findings suggest that this non-coding small RNA gene is an important regulator of male development controlled by cross-talk between the genetic networks and the environmental conditions.

THE CONSENSUS OF CMS, EGMS, AND GMS

Mutants from various plant species allow us to use genetic strategies to uncover the developmental mechanisms of the male reproductive system (Wilson and Zhang, 2009; Borg and Twell, 2010; Twell, 2011). In Arabidopsis many important factors have been identified, and hence for this species, researchers have constructed relatively comprehensive pathways involved in the development of the male reproductive system (Wilson and Zhang, 2009; Twell, 2011). These Arabidopsis mutants were mainly produced by physical and chemical mutagens, such as X-rays, EMS, etc. Because the male sterility phenotypes of these mutants were caused by genomic mutation, we refer to this as GMS. Although differences in the reason for male sterility between these systems exist, CMS or EGMS and GMS might share a consensus in nucleus gene regulation (**Figure 1**).

In CMS plants, CMS-related proteins in mitochondria make retrograde signals to activate nuclear factors to direct downstream pathways defining pollen fate. For EGMS, the external environmental signals, light or temperature, are received by unknown receptors, and then transmit to the nucleus to guide pollen development. Therefore, as the phenotype of these two kinds of male sterility was both finally executed by the nucleus, the signals to cause male sterility in CMS and EGMS must impair the normal pathways that spatio-temporally regulate the development of the male reproductive system. In the mutants of male sterility (GMS), the normal pathways have been deteriorated by missing some key factors. From this perspective, the study of GMS occupies a core position for comprehensively understanding male sterility, including both CMS and EGMS.

The development of the male reproductive system of rice is a very complex process (**Figure 2**) which includes the formation of the stamen with differentiated anther tissues, in which microspores/pollens are generated, which is followed by anther dehiscence and subsequently by pollination (Goldberg et al., 1993) (**Figure 2** not show the stamen formation process).

NETWORK OF MALE REPRODUCTIVE SYSTEM DEVELOPMENT

Benefited from the identification of a number of male-sterile mutants in rice, a preliminarily regulatory network of pollen exine formation and tapetal development can be constructed (**Figure 3A**). *OsMADS3* and *OsMADS58* which belong to C-class MADS box transcription factor family were identified to control archesporial specification and the start of anther development (Yamaguchi et al., 2006). The phenotype of the MULTIPLE SPOROCYTE1 (*msp1*) mutant shows increased numbers of male and female sporocytes and disrupted anther wall layers. *MSP1* which encodes a LRR receptor-like kinase (LRR-RLKs) might play the function of initiating anther wall development and controlling male and female sporogenesis (Nonomura et al., 2003). *OsTPL1A* was found to bind *MSP1* to co-regulate sporogenesis (Zhao et al., 2008), and the interaction between them was identified by yeast two hybrid studies. BAM1 and BAM2, which

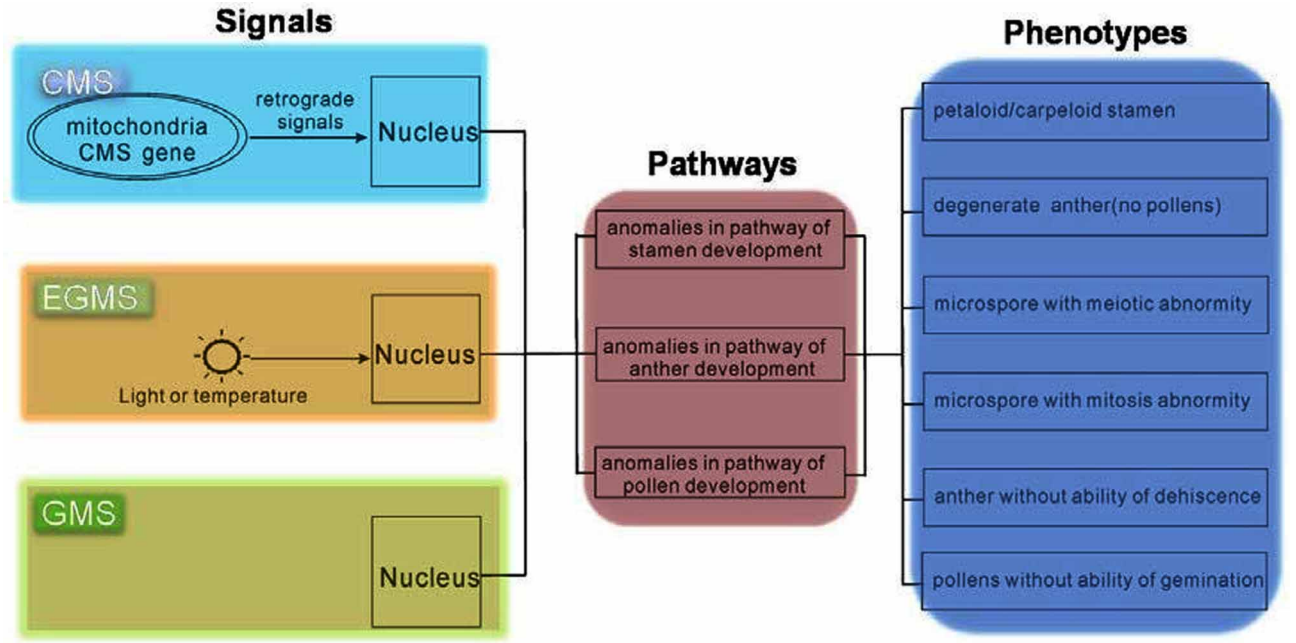

FIGURE 1 | Consensus in regulatory pathways of CMS, EGMS, and GMS. Although the original signals of CMS, EGMS, and GMS are different, the pathways and phenotypes might be the same.

also belong to the LRR-RLKs protein family, were found to act redundantly in defining the parietal cells that give rise to the endothecium, middle and tapetal cell layer, as these cell layers disappeared in the double mutant *bam1bam2* (Hord et al., 2006). *OsUDT1* which encodes a basic helix-loop-helix (bHLH) protein seems to be one of the earliest players in tapetal development (Jung et al., 2005). *OsTDR*, a possible regulator downstream of *OsUDT1*, controls tapetum development. It also encodes a bHLH protein and has been proposed as a trigger for rice tapetum PCD. The TAPETUM DEGENERATION RETARDATION (*tdr*) mutant has highly vacuolated and retarded PCD tapetum and degenerated microspores (Li et al., 2006). Another two genes, *OsCP1* and *Osc6* (Zhang et al., 2010a), which respectively encode a cysteine protease and a lipid transfer protein (*LTP*), were identified by chromatin immunoprecipitation-PCR and electrophoretic mobility shift assay (EMSA) as direct downstream targets of OsTDR protein. Recently, APOPTOSIS INHIBITOR5 (API5) and its two partners AIP1 and AIP2 were also found to bind the promoter of *OsCP1* and increase its expression level. They regulate tapetal PCD through regulating the expression of *OsCP1* (Li et al., 2011b). Through studying pollen exine, researchers have found *GAMYB* which is downstream of The PERSISTANT TAPETAL CELL1 (*PTC1*). The *GAMYB also* acts downstream of *MSP1-OsTDLIA* which is supported by the fact that down-regulation of *GAMYB* was observed in *msp1-4* anthers (Wang et al., 2006a). *PTC1* functions downstream of *GAMYB* (Aya et al., 2009) but on the same level as *TDR* in controlling anther and pollen development. Interestingly, the *TDR*, *OsCP1,* and *OsC6* that take charge of Tapetal PCD were found being regulated by *GAMYB* and *PTC1*, indirectly or directly. Meanwhile, the recently identified genes *CYP703A3*, *CYP704B2,* and *RAFTIN* that are involved in lipid metabolism and transport during pollen exine formation might act downstream of *GAMYB, PTC1,* and *TDR* (Li et al., 2010, 2011a). The data also show that *CYP704B2* and *RAFTIN* might be affected by *TDR* (Li et al., 2011a).

In contrast, the regulatory model to describe how cell cycle-associated genes control the process that diploid pollen mother cells undergo in meiosis and mitosis to produce mature pollen has also been preliminarily constructed in *Arabidopsis* (**Figure 3B**). Considering the high conservation of these genes, studies of their homologs in rice would probably lead to similar results, although many studies need to be performed in rice to confirm this.

ENGINEERED MALE STERILITY

Conventional breeding methods utilizing naturally occurring mutations to create male sterility have their shortcomings including: being time-consuming, the limitation of genotype numbers and the uncertainty of targets. However, we consider it would be possible to construct inducible male-sterile plants based on our design, because modern biotechnology has offered us new approaches to obtain male-sterile plants. Because of limitations in propagating male-sterile plants without segregations in the next generation or insufficient restoration of fertility for fruits or seed-setting of the hybrid varieties, only one engineered system is, so far, used in practical breeding. This exploration for engineering male sterility rice started as early as the 1990's when Ling et al. (1998) successfully developed completely sterile rice plants by co-transferring Barnase-ps1 and pHcintG genes into genomic DNA of the rice variety Tai-Pei-309.

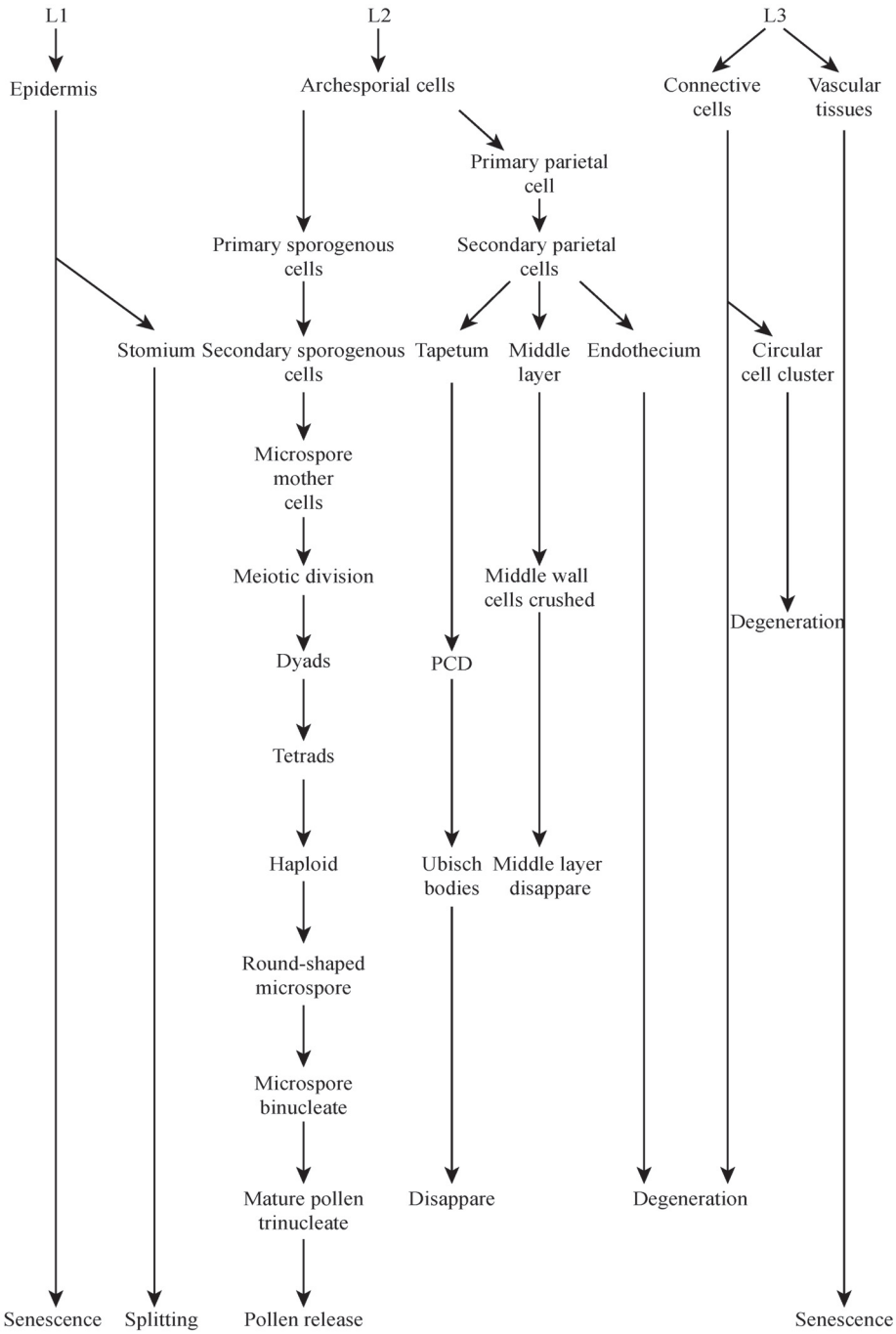

FIGURE 2 | Major events during rice microspores development and dehiscence. Development stages and events were taken from Zhang et al. (2011). Some events were marked with boxes. L1, L2, L3 means layer 1, layer 2, and layer 3, respectively.

Based on the target location of the transformed alien genes, the engineered male-sterile lines can be classified into two types: cytoplasmic male-sterile and nuclear male-sterile lines. Relative to the engineered nuclear male-sterile lines, development of engineered cytoplasmic male-sterile lines is more difficult due to the obstacle of transforming the target genes directly into mitochondrial or chloroplast genomes. Daniell et al. (2002) successfully introduced alien genes into the chloroplast genome, and obtained completely male-sterile plants with a novel method, and this method has been further successfully applied in *phaA* transformation research (Ruiz and Daniell, 2005). In this way, these researchers opened the door to breed environmentally friendly genetically modified rice sterile lines by preventing the diffusion of seeds or pollen grains containing alien genes into the environment.

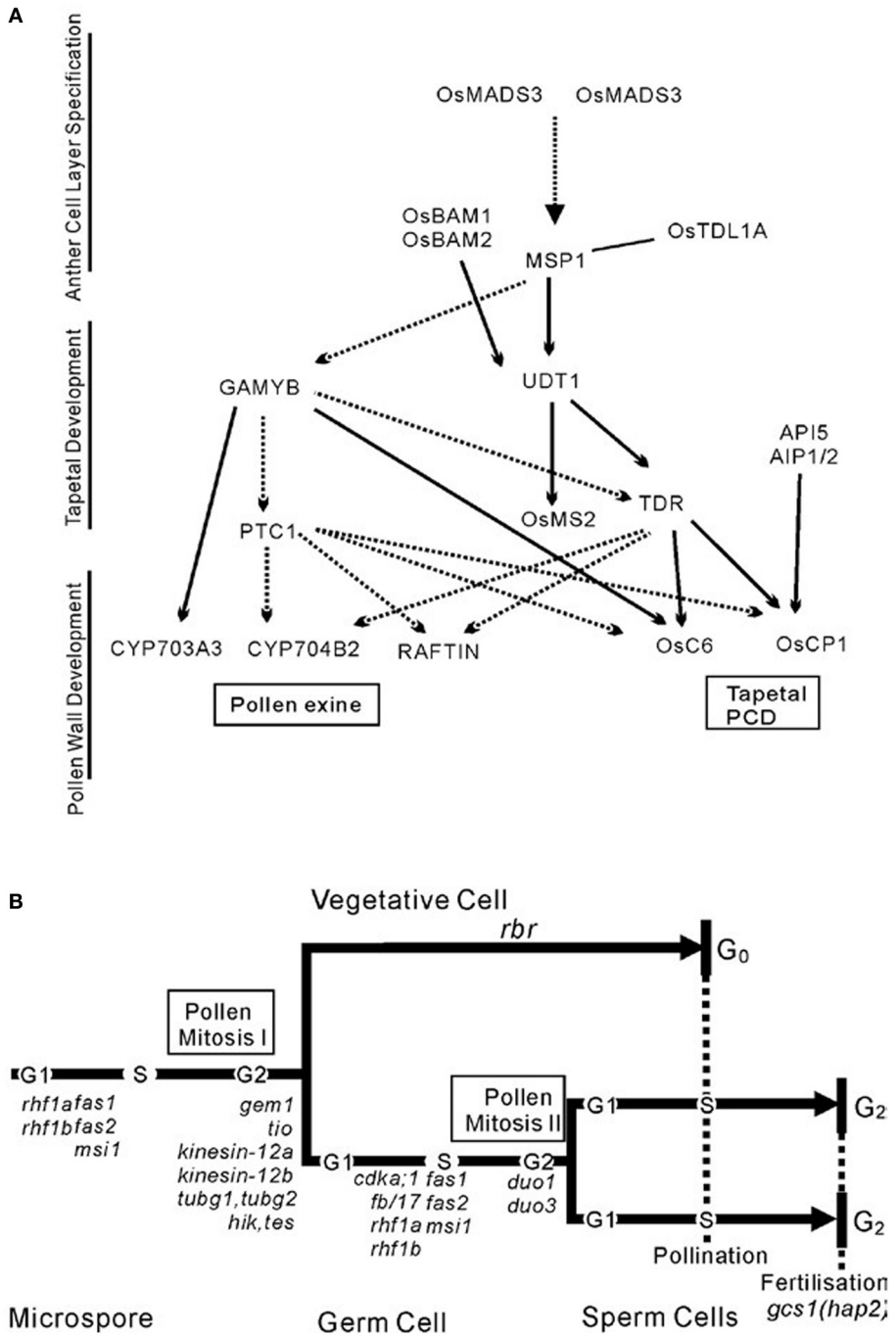

FIGURE 3 | Two local pathways of development of the male reproductive system. (A) The networks of pollen development in rice. **(B)** Schematic illustrating the cell lineages and cell cycle progression in microspore development in the context of gametophytic mutations that affect asymmetric division, cell cycle progression, and patterning. This schematic is from Twell (2011).

Pollen development is controlled by a sequential expression of genes specially expressed in reproductive tissues, and is highly susceptible to the cellular environment. Any imbalance of the expression of genes related to anther and microspores development, such as starch synthesis, nutrition transportation, or energy production will lead to abnormalities and disable male gametogenesis. This provides the theoretical possibility of EGMS or GMS breeding. *PAIR1* (Nonomura et al., 2004) controls homologous chromosome pairing and cytokinesis in male and female meiocytes of rice. *WDA1* (Jung et al., 2006) shows a pivotal role in the biosynthesis of very-long-chain fatty acids in both outer and inner layers of anthers. Both of their mutants

showed abortive pollens. In addition, the male-sterile phenotypes of mutants of *MSP1* (Nonomura et al., 2003), *MADS3* (Hu et al., 2011), *API5* (Li et al., 2011b), *UDT1* (Jung et al., 2005), and *WDA1* (Jung et al., 2006) which have been mentioned above given us evidences that plants would show male-sterile phenotypes if any of these genes were destroyed or silenced.

Theoretically, we could acquire male-sterile plants by disturbing the expression of any genes that were identified to take part in anther or pollen development. To date, very few of them have been successfully applied in practical hybrid rice breeding. Chen et al. (2007) used the *Ubiqutin* promoter to over-expressed UDP-glucose pyrophosphorylase 1 (*Ugp1*) which plays critical roles in rice growth, especially in pollen development. Interestingly, the attempt to over-express *Ugp1* actually silenced *Ugp1* and unexpectedly acquired a novel thermo-sensitive genetic male sterility rice. Pollen mother cells of *Ugp1*-silenced plants appeared normal before meiosis, but during meiosis normal callose deposition was disrupted. Consequently, the pollen mother cells began to degenerate at the early meiosis stage, eventually resulting in complete pollen collapse. This finding might give us a novel way to practical breeding of EGMS lines in rice using genes that are in the network of male reproductive development.

Engineered nuclear male sterility controlled by a single gene is easy to handle from the angle of using current molecular techniques, but a problem with these nuclear MALE-STERILE transformants is that they segregate for male fertility or sterility and must be over planted and rogued by hand or sprayed with herbicides to remove male-fertile plants (Ruiz and Daniell, 2005). This confines the application of engineered MALE-STERILE lines in a simple way for commercial production. Multiple gene controlled MALE-STERILE lines may help us avoid such insurmountable issues. Recently, Kubo et al. (2011) identified a novel sterility locus on rice chromosome 2 named Epistatic Factorfor S24 (EFS). EFS interacts with S24, a gene for indica and japonica hybrid male sterility, and shows a recessive sporophytic allele in japonica rice. When S24 is in combination with the homozygous japonica EFS allele (efs-j), male sterility will occur, while the indica allele (EFS-i) can dominantly counteract the pollen sterility caused by S24 heterozygosity. These results demonstrate that an additional epistatic locus is an essential element in the hybrid sterility caused by allelic interaction at a single locus in rice. If we combined these two epistatic loci with other phenotype markers, it will provide a possibility to employ allelic interaction of two loci in two different rice varieties to develop a robust GMS system for hybrid rice.

Alternatively, some novel male-sterile system created in other crops may be able to be applied in rice breeding practice. Zhang et al. (2010b) developed a novel system for creating male-sterile transgenic plants by down-regulation of the specifically expressed glyphosate tolerance CP4 EPSPS gene in male reproductive tissues. The CP4 EPSPS gene was driven by a modified CaMV 35S promoter with three tetracycline operator copies in the vicinity of the TATA box. The controllable transgenic plants could tolerate exposure of glyphosate but the male tissue was sensitive. The novel inducible male sterility system is applied and easy to handle, and it might be applicable to a wide range of crop plants including rice. Meanwhile, Engelke et al. (2010) constructed a male sterility and restoration system by interference with extracellular invertase activity in potato. Antisense repression of the anther-specific cell wall invertase or interference with invertase activity by expressing a protein inhibitor under the control of the anther-specific invertase promoter results in a block during early stages of pollen development, thus causing male sterility without any pleiotropic effects. Restoration of fertility was successfully achieved by substituting the down-regulated endogenous plant invertase activity by a yeast invertase fused to the N-terminal portion of potato-derived vacuolar protein proteinase II (PiII–ScSuc2), under control of the orthologous anther-specific invertase promoter *Nin88* from tobacco. The chimeric fusion PiII–ScSuc2 is known to be *N*-glycosylated and efficiently secreted from plant cells, leading to its apoplastic location. Furthermore, the *Nin88::PiII-ScSuc2* fusion does not show effects on pollen development in the wild-type background. Thus, such plants can be used as paternal parents of a hybrid variety, thereby the introgression of *Nin88::PiII-ScSuc2* to the hybrid is obtained and fertility is restored. Because of the conservative of plant invertases, this system could also be used in rice.

PROSPECT

An increasing number of genes causing GMS have been identified by forward or reverse genetic strategies and can be integrated into some preliminary network systems (**Figure 3**). However, currently the in-depth study in this field to explore the network is confined by limited genetic materials. After all, the number of mutants is still relatively few compared to the number of genes in rice nuclear genome. Comprehensive resolution of the spatio-temporal profiles of key component of the male reproductive system at the transcriptomic and proteomic levels will greatly facilitate our understanding of the mechanisms of male reproductive development. Previous studies have accumulated large amount of transcriptomic (Becker et al., 2003; Lee and Lee, 2003; Honys and Twell, 2004; Pina et al., 2005; Borges et al., 2008; Haerizadeh et al., 2009; Tang et al., 2010; Wei et al., 2010; Hafidh et al., 2012) and proteomic (Mayfield et al., 2001; Chen et al., 2006; Dai et al., 2006, 2007; Sheoran et al., 2007; Pertl et al., 2009; Han et al., 2010; Lopez-Casado et al., 2012) data on development of pollen grains, but there are still a lot of blanks to fill in. For example, the proteomic study of post-translational modification, phosphorylation, and ubiquitination, etc., during the process is still limited (Mayank et al., 2012). In addition, epigenetic regulation by small non-coding RNAs and modification of DNA and histone has emerged for with roles in male reproductive system development (Johnson and Bender, 2009; Slotkin et al., 2009). However, with the great improvements in RNA and protein detection technology, such as single molecular sequencing (Fang et al., 2012) and label-free MS (Levin and Bahn, 2010), and combined with laser capture microdissection technology, we would have the ability to achieve more subtle spatio-temporal expression profiles with less tissue. In this way even sperm and vegetative cells that it was previously impossible to study with omic data, can become targets for studies.

Moreover, the study of how CMS and EGMS rice receive and transfer mitochondrial retrograde signals and light/temperature

signals is another key and interesting aspect for male sterility. In HL-CMS, verifying the hypothesis that the level of ATP and ROS may play the retrograde signals from mitochondrion to nucleus and how the nucleus genes receive and transfer the signals needs further study. In PGMS, how the lncRNAs (pms3) sense the different photoperiod and alter their expression is also very interesting and awaits much deeper research.

Taken together, concerted efforts from multiple angles would pave the road to rapid progress in understanding the complex regulatory networks and finally enable us to attain a holistic concept for the development of rice male reproductive system. All the data harvested in these studies will definitely help us to freely manipulate fertility in rice and other crop plants to facilitate hybrid breeding in the future.

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Proteomic evaluation of genetically modified crops: current status and challenges

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Hectares of genetically modified (GM) crops have increased exponentially since 1996, when such crops began to be commercialized. GM biotechnology, together with conventional breeding, has become the main approach to improving agronomic traits of crops. However, people are concerned about the safety of GM crops, especially GM-derived food and feed. Many efforts have been made to evaluate the unintended effects caused by the introduction of exogenous genes. "Omics" techniques have advantages over targeted analysis in evaluating such crops because of their use of high-throughput screening. Proteins are key players in gene function and are directly involved in metabolism and cellular development or have roles as toxins, antinutrients, or allergens, which are essential for human health. Thus, proteomics can be expected to become one of the most useful tools in safety assessment. This review assesses the potential of proteomics in evaluating various GM crops. We further describe the challenges in ensuring homogeneity and sensitivity in detection techniques.

Keywords: genetically modified crops, biological safety, unintended effects, substantial equivalence, proteome and proteomics

INTRODUCTION

Genetic modification has become the fastest-adopted technology in the history of modern agriculture. It involves the transfer of individual genes that encode specific desirable traits into the host, thus producing genetically modified (GM) crops (also transgenic crops) (Garcia-Canas et al., 2011). GM crops therefore possess improved agronomic traits, such as resistance to insects, tolerance to herbicides, improved productivity and quality, and other traits not present before genetic modification.

The hectares of GM crops continue to greatly increase worldwide (James, 2010; D'Alessandro and Zolla, 2011). Many important crops have been GM by transgenes; examples are maize (*Zea mays*), potato (*Solanum tuberosum*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), and cotton (*Gossypium hirsutum*). However, genetic modifications generally represent a double-edged sword. Besides inducing desired traits, modifications in a plant genome might result in unintended effects, which may affect human health or the environment (Ioset et al., 2007). Some unintended effects may result from integration of the transgene and/or biological interactions caused by transgene-encoding proteins, which can be predicted by knowledge of transgene integration sites, transgene function and transgene-related metabolic pathways. However, transgene integration and/or transformation and tissue culture during transgenic progress may induce unintended genomic alterations in GM plants such as deletions, insertions, and rearrangements, which may generate secondary or pleiotropic effects (Kuiper et al., 2001; Cellini et al., 2004; Garcia-Canas et al., 2011). The unintended effects associated with genomic alterations are unpredictable (Garcia-Canas et al., 2011). With the commercialization of GM crops, these unintended effects are one of the most controversial issues in debating the biological safety of GM crops. A systematic comparative analysis of molecular features of GM crops and their comparators is needed to clarify unintended effects (Cellini et al., 2004; Garcia-Canas et al., 2011).

The concept of "substantial equivalence" was proposed as a cornerstone of biological safety assessment in many countries (OECD, 1993). Although "the principle left much scope for individual (and national) interpretation" (Kok and Kuiper, 2003), it is still an acceptable standard to evaluate the biological safety of GM crops. The increasingly use of "omics" technologies, including genomics, proteomics, and metabolomics, in GM crops analysis has provided important information on the molecular characteristics of GM crops and extended our understanding of the biological safety of GM crops. In this mini-review, we briefly discuss technologies used in evaluating GM crops, and summarize current proteomics insights into GM crops.

TECHNOLOGIES FOR EVALUATING GM CROPS

Targeted analysis is the primary method used for evaluating GM crops. According to the Organisation for Economic Co-operation and Development (OECD, 2006), 50–100 or more analytes are selected for targeted analysis of each crop variety. These analytes typically represent a limited number of crop compositions; they cannot cover unknown toxins, antinutrients, or other secondary products that could result from the genetic modification (Kuiper et al., 2001). Accordingly, targeted analysis is useful for detecting primary or intended but not unintended effects of genetic modification and has been considered biased (Millstone et al., 1999).

Profiling techniques have emerged as useful approaches to evaluate the unintended effects. They allow for simultaneous

characterization and comparison of the genome, proteome, and metabolome of an organism, thus increasing the chances of detecting the unintended effects (Kuiper et al., 2003; Ruebelt et al., 2006). These non-targeted approaches have become more comprehensive than targeted analysis in characterizing the composition and performance of GM crops and detecting the unintended effects.

Transcriptome profiling has been used to characterize several GM crops, including maize, barley and rice (Coll et al., 2008, 2009; Kogel et al., 2010; Montero et al., 2011). Transcriptome profiling could be used to investigate gene expression changes in GM crops and has potential to detect unintended effects. But changes in a transcriptome do not necessarily lead to changes in a proteome or metabolome, therefore do not necessarily predict changes in food composition and quality (Chassy, 2010), so transcriptomic profiling is limited in evaluating unintended effects.

Besides transcriptomic techniques, proteomic and metabolomic methods are two complementary tools for evaluating GM crops. In recent years, metabolomic analysis has become prevalent (Catchpole et al., 2005; Baker et al., 2006; Garcia-Villalba et al., 2008; Kim et al., 2009). Because of its ability for use in analyzing a great number of metabolites and reducing the cost of analysis for each analyte, metabolomics has been considered a replacement for conventional compositional analysis (Chassy, 2010). However, this method is only capable of measuring hundreds of metabolites, not the thousands of metabolites in a plant (Davies, 2010). Furthermore, differences in metabolomic methodologies, data analysis, and statistical analysis have resulted in less reproducibility. Therefore, metabolomics may not be advantageous for safety assessment (Chassy, 2010). Proteins are key players in gene function and are directly involved in metabolism and cellular development, thus forming the central bridge between the transcriptome and metabolome (Salekdeh and Komatsu, 2007). Furthermore, proteins have roles as toxins, antinutrients, or allergens, which have great impact on human health. Therefore, proteomic studies would provide important information for understanding changes in biological processes after genetic modification and are important for evaluating biological safety of GM crops.

PROTEOMIC CHARACTERISTICS OF GM CROPS

2-D gel electrophoresis (2-DE) analysis, established in 1975, revolutionized the study of proteins (Klose, 1975; O'Farrell, 1975) and has helped in the development of proteomics. With use of immobilized pH gradients (Bjellqvist et al., 1982) and mass spectrometry (MS), proteomics has played important roles in understanding the mechanisms and protein–protein interaction networks underlying biological processes. Comparative proteomic strategies combined with 2-DE and MS and with liquid chromatography tandem mass spectrometry (LC-MS/MS) have been extensively used to evaluate the effects of genetic modification on the proteomes of eight GM crops: maize, pea, potato, rice, soybean, tobacco, tomato, and wheat (**Table 1**). These studies involved safety evaluation of GM crops and functional characterization of GM crops. Some studies investigated different varieties for detecting natural variation. Proteomic evaluation of a GM pea cultivar carrying α-amylase inhibitor-1 (αAI1) was discussed elsewhere (Chen et al., 2009; Islam et al., 2009; Ricroch et al., 2011). An excellent review discussed MS-based identification of transgenic proteins in GM crops (Garcia-Canas et al., 2011). So here we mainly discuss the recent status of proteomic evaluation of the other seven GM crops.

MAIZE

GM maize is the second most commercialized GM crop worldwide and has been approved for release in many countries (Coll et al., 2008). MON810 is insect-resistant GM maize produced by inserting a truncated form of the *cry1Ab* gene from *Bacillus thuringiensis* in the maize genome. The proteomes of MON810 varieties and their comparators have been well studied because of their commercial importance (Albo et al., 2007; Zolla et al., 2008; Barros et al., 2010; Coll et al., 2011). Albo et al. (2007) compared the seed proteomes of MON810 variety BT (derived by crossing La73-Bt and La17-Bt) and the near-isogenic control (derived by crossing La73 and La17) and detected differences in six proteins: glucose and ribitol dehydrogenase appeared specific to BT, and endochitinase A to the control, with a difference in expression of triosephosphate isomerase 1, globulin-1 S, cytosolic 3-phosphoglycerate kinase, and aldose reductase between BT and the control. Coll et al. (2011) found a few spots with 1.1- to 1.8-fold change in expression in seed proteomes of two other sets of MON810 varieties, PR33P67, and DKC6575, and their corresponding non-GM controls, PR33P66, and Tietar. The two studies showed that the proteomes of these GM varieties should be virtually identical to that of their comparators. However, Zolla et al. (2008) found that proteomes of seeds from PR33P67 and PR33P66 had 43 differentially expressed proteins (DEPs) possibly caused by the transgene. This situation was probably resulted from different cultured conditions of materials (agriculture field vs. environmentally controlled growth chamber). Barros et al. (2010) evaluated the effect of growing season, growing location, and transgenes on transcriptomes, proteomes, and metabolomes of maize seeds using the GM lines DKC78-15B (hybrid of event MON810) and DKC78-35R (hybrid of event NK603) and the near-isogenic non-GM hybrid variety CRN3505. Transgenes produced less variation in transcript, protein, or metabolite profiles of each sample pair than did environmental factors. Balsamo et al. (2011) compared leaf proteomes of four MON810 varieties and their controls using the same 2-DE-based proteomics and revealed seven DEPs in the DKBYG240–DKB240 pair, five in the DKBYG350–DKB350 pair, and none in the other two GM–non-GM pairs. Thus, the leaf proteomes of four MON810 varieties may have been similar to those of their non-GM counterparts. Together, these data suggested that the expression of transgenes in host maize plants had no significant effects on proteome of the host, and evaluating the molecular characteristics of GM crops should involve environmental factors.

POTATO

Potato is the fourth major crop widely grown worldwide. Despite diverse potato varieties with desired traits, GM potato is still

Table 1 | Use of proteomic techniques to evaluate genetically modified crops.

Crop	Donor organism	Transgenic protein/*gene*	Traits	Tissues	Purpose	Proteomic approaches	References
Maize	*Bacillus thuringiensis*	CryIA(b)/*cry*IA(b)	Insect resistance	Seeds	Detection of unintended effects	2-DE, MALDI-TOF MS	Albo et al., 2007
	Bacillus thuringiensis	CryIA(b)/*cry*IA(b)	Insect resistance	Seeds	Detection of unintended effects	2-DE, nLC-ESI-IT MS/MS	Zolla et al., 2008
	Bacillus thuringiensis	CryIA(b)/*cry*IA(b)	Insect resistance	Seeds	Detection of unintended effects	2-DE, ESI-IT MS/MS	Coll et al., 2011
	Bacillus thuringiensis	CryIA(b)/*cry*IA(b)	Insect resistance	Leaves	Detection of unintended effects	2-DE, MALDI-TOF MS	Balsamo et al., 2011
	Bacillus thuringiensis	CryIA(b)/*cry*IA(b)	Insect resistance	Seeds	Detection of unintended effects	2-DE, no identification	Barros et al., 2010
	Agrobacterium tumefaciens	CP4 EPSPS/*cp4 epsps*	Glyphosate tolerance				
Pea	Bean (*Phaseolus vulgaris*)	α-amylase inhibitor-1/*αai1*	Pea weevil resistance	Seeds	Detection of unintended effects	DIGE, ESI Q-TOF MS/MS	Islam et al., 2009
	Bean	α-amylase inhibitor-1/*αai1*	Pea weevil resistance	Seeds	Detection of unintended effects	2-DE, MALDI-TOF MS/MS	Chen et al., 2009
Potato	Potato	Antisense G1-1 gene/*G1-1*	Sprouting delay	Tubers	Functional characterization of GM crops	MALDI-TOF MS	Careri et al., 2003
	Aureobasidium pullulans	Glucan branching enzyme/*W2*	Waxy phenotype	Tubers	Detection of unintended effects	2-DE, μLC-ESI-IT MS/MS, μLC-ESI-QqTOF MS/MS	Lehesranta et al., 2005
	Potato	Glycoprotein/*Mal1*	Changes in cell wall structure				
	Potato	AdoMetDC/*SamDC*	Modified metabolism				
	Tomato	Cathepsin D inhibitor/sl*cdi*	n	Leaves	Functional characterization of GM crops	2-DE, SELDI TOF MS	Goulet et al., 2010
	Tomato	Cathepsin D inhibitor/sl*cdi*	n	Tubers	Detection of unintended effects	2-DE, LC-ESI-IT MS/MS	Khalf et al., 2010
Rice	Rice	YK1/YK1	Several stress tolerance	Cultured cells	Functional characterization of GM crops	2-DE, internal amino acid sequencing	Takahashi et al., 2005
	Human (*Homo sapiens*)	hGM-CSF/*hgm-csf*	hGM-CSF reactor	Seeds	Functional characterization of GM crops	iTRAQ, nLC ESI-QTOF MS/MS	Luo et al., 2009
	Streptomyces hygroscopicus	PAT/*bar*	Herbicide resistance	Seeds	Detection of unintended effects	DIGE, MALDI-TOF MS	Gong et al., 2012
	Bacillus thuringiensis/cowpea	CryIAc/cowpea trypsin inhibitor/*cry1Ac/sck*	Insect resistance	Seeds			

(Continued)

Table 1 | Continued

Crop	Donor organism	Transgenic protein/*gene*	Traits	Tissues	Purpose	Proteomic approaches	References
	Sunflower (*Helianthus annuus*)	SSA/SSA	n	Seeds	Functional characterization of GM crops	2-DE, MALDI-TOF MS	Islam et al., 2005
	Rice	thaumatin-like protein/thaumatin-like gene	Bacterial blight disease resistance	Leafs	Functional characterization of GM crops	2-DE, N-terminal amino acid sequencing, MALDI-TOF MS	Mahmood et al., 2009
Soybean	*Agrobacterium tumefaciens*	CP4 EPSPS/*cp4 epsps*	Glyphosate tolerance	Seeds	Optimization of parameters	2-DE, MALDI-QTOF MS	Brandao et al., 2010
	Agrobacterium tumefaciens	CP4 EPSPS/*cp4 epsps*	Glyphosate tolerance	Seeds	Functional characterization of GM crops	2-DE, DIGE, MALDI-QTOF MS, MALDI-QTOF MS/MS, ESI-QTOF MS/MS	Barbosa et al., 2012
	Agrobacterium tumefaciens	CP4 EPSPS/*cp4 epsps*	Glyphosate tolerance	Seeds	Functional characterization of GM crops	2-DE, LC-MS/MS	Batista et al., 2007
Tobacco	*Arabidopsis thaliana*	AdoMetDC/*AdoMetDC*	Perturbed polyamine metabolism	Leaves	Functional characterization of GM crops	2-DE, MALDI-TOF MS, ESI-QTOF MS/MS	Franceschetti et al., 2004
	Tomato	Prosystemin/Prosystemin gene	Insect resistance	Leaves	Detection of unintended effects	2-DE, MALDI-TOF MS, μLC-ESI-IT MS/MS	Rocco et al., 2008
	n	ScFv B9/scFv B9	Virus resistance	Leaves	Detection of unintended effects	DIGE, MALDI-TOF MS, μLC-ESI-IT MS/MS	Di Carli et al., 2009
Tomato	Tomato spotted wilt virus	TSWV nucleoprotein/*TSWV-N*	Virus resistance	Seeds	Detection of unintended effects	2-DE, MALDI-TOF MS	Corpillo et al., 2004
	n	ScFv G4/scFv G4	Virus resistance	Leaves	Detection of unintended effects	DIGE, MALDI-TOF MS, μLC-ESI-IT MS/MS	Di Carli et al., 2009
	n	ScFv G4/scFv G4	Virus resistance	Leaves	Functional characterization of GM crops	DIGE, μLC-ESI-IT MS/MS	Di Carli et al., 2010
Wheat	Tobacco	Rab-1/*rab*-1	Improved functional properties	Seeds	Detection of unintended effects	2-DE, MALDI-TOF MS, nESI-QqTOF MS/MS	Di Luccia et al., 2005
	Streptomyces hygroscopicus	PAT/*bar*	Herbicide resistance	Seeds	Functional characterization of GM crops	2-DE, MALDI-TOF MS, MALDI-IT MS/MS	Horvath-Szanics et al., 2006
	Wheat	LMW-GS/LMW-GS gene	Improved functional properties	Seeds	Functional characterization of GM crops	2-DE, LC-ESI-QTOF MS/MS	Scossa et al., 2008

n, no information.

an important topic in potato breeding. Comparative proteomics was used to evaluate variation in tuber proteomes of a large collection of potato genotypes, including different varieties, landraces, and GM lines (Lehesranta et al., 2005). Different varieties and landraces showed clear separations, which indicates extensive genotypic variation; but the differences between GM and non-GM lines were less significant (Lehesranta et al., 2005). A proteomic study by Careri et al. (2003) showed that spatial sites of transgene-encoding proteins affected the proteome of the analyzed sample—GM potato PG50 carrying the antisense *G1-1* gene of unknown function. Phenotypic and expression analysis confirmed that *G1-1* was highly expressed in apical eyes, and its expression regulated transition from dormancy to sprouting tubers (Agrimonti et al., 2000; Marmiroli et al., 2000). A proteomic analysis revealed no difference between PG50 and non-GM lines with use of whole tubers but several differences with use of only apical eyes. This finding was consistent with the expression of *G1-1* gene in apical eyes. Other studies found inconsistent proteome changes in different tissues of GM potato (Goulet et al., 2010; Khalf et al., 2010). Transgenic potato expressing cathepsin D inhibitor (S/CDI) showed significant variation in leaf proteome as compared with the control (Goulet et al., 2010) but no quantitative proteome difference in tubers of the two samples (Khalf et al., 2010). Because antisense *G1-1* in PG50 affects only the proteome of tissue expressing antisense *G1-1*, the different effect of the sl*cdi* transgene on leaf and tuber proteomes may result from different expression of the gene in leaves and tubers, although the two studies did not explore the different expression. Therefore, examining expression patterns and levels of transgenes is important to explain proteomic data.

RICE

Rice feeds one-quarter of world's population and is one of main crops in the world (Agrawal and Rakwal, 2011). It has become model system to study biological issues. GM rice has been quickly developed although commercialization of GM rice is still in debate. Gong et al. (2012) evaluated proteome differences in seeds from two sets of GM rice (Bar68-1 carrying *bar* and 2036-1a carrying *cry1Ac/sck*) and their controls by 2-DE differential in-gel electrophoresis (2D-DIGE). To obtain relatively objective data, this study included other rice varieties to evaluate proteome variations related to spontaneous genetic variation, genetic breeding, and genetic modifications. GM events did not substantially alter protein profiles as compared with conventional genetic breeding and natural genetic variation (Gong et al., 2012). However, use of GM rice expressing human granulocyte-macrophage colony stimulation factor and shotgun analysis of trypsin digests labeled by isobaric tags for relative and absolute quantitation (iTRAQ) revealed more DEPs (103 proteins) between GM rice and wild-type rice endosperm (Luo et al., 2009). No parallel experiments have compared the output of the two proteomic strategies with the same set of GM rice and control, so explaining these differences is difficult. One explanation may be that the LC-MS/MS shotgun approach provides more comprehensive identification of proteins. In addition, different exogenous genes and/or different insert events of these genes in host genomes may have different impacts on the host crops. Indeed, several proteomic studies of GM rice have investigated biological changes with exogenous gene insertion in seeds (Islam et al., 2005), cultured cells (Takahashi et al., 2005), and leaf blades (Mahmood et al., 2009). A final explanation may be that these transgene-encoding proteins accumulate at different levels in analyzed tissues, for a differential effect on proteome profiling of the analyzed tissues, as we discussed previously for GM potato. Therefore, the safety assessment of GM crops should be case by case.

SOYBEAN

Soybean is one of the main foods responsible for allergic reactions worldwide, but GM soybean remains one of the most commercialized GM crop. Batista et al. (2007) compared seed proteome of Roundup Ready soybean carrying *cp4 epsps* and non-transgenic control; they found that soybean endogenous allergen expression did not seem to be altered after genetic modification. Brandao et al. (2010) evaluated variables that affect the analysis of protein profiles before comparative proteomic analysis of Roundup Ready soybean (MSOY7575RR) and a non-GM comparator (MSOY7501). Optimal parameters were manual image editing, 300-μg protein loading for 3–10 pH range strips and 500-μg loading for 4–7 pH strips. The authors identified 10 DEPs between seed proteomes via 2-DE with both pH 3–10 and 4–7 gel strips. Barbosa et al. (2012) used a more sensitive 2D-DIGE technique to further compare protein profiles of seeds from the same sample pair and revealed only four DEPs with pH 4–7 strips, of which only one DEP was identical to that identified by Brandao et al. (2010). The two studies used the same materials and protein extraction methods, so the inconsistent results probably derived from standards for judging DEPs in comparative proteomics. Barbosa et al. (2012) identified DEPs based on statistical analysis ($p \leq 0.05$, by Student t-test) and fold change in expression ($\geq$1.5, 50% variation), but Brandao et al. (2010) identified DEPs by only fold change in expression ($\geq$1.8, ~90% variation). Therefore, statistical analysis may be important to determine differential expression of proteins.

TOBACCO

Tobacco is of great economic importance, despite hazards and environmental problems accompanying its production. Di Carli et al. (2009) used 2D-DIGE to compare the leaf proteomes of GM tobacco expressing ScFv (B9) antibody against the G1 envelope glycoprotein of tomato spotted wilt virus (TSWV) and a non-GM control. The recombinant antibody did not significantly affect leaf protein profiles. However, a proteomic study of tobacco expressing tomato prosystemin showed that expression of the gene greatly changed leaf protein profiles of the host (Rocco et al., 2008). The two studies also indicated the need for evaluating substantial equivalence in GM plants on a case-by-case basis. Furthermore, changes in host proteome are associated with the expression level of a transgene, as shown in the study of GM tobacco lines overexpressing S-adenosylmethionine decarboxylase (AdoMetDC) (Franceschetti et al., 2004). A comparison of leaf proteomes from three GM tobacco lines with different levels of AdoMetDC revealed that as compared with the control, the proteome of the GM line with the highest level of AdoMetDC had the largest numbers of DEPs and that

of the line with the lowest level of AdoMetD had the lowest numbers of DEPs.

TOMATO

Tomato has great economic and nutritional value. Genetic modifications of tomato mainly involved virus resistance, and delayed fruit ripening. Corpillo et al. (2004) first assessed the substantial equivalence of GM tomato using proteomic approaches. The authors found no qualitative or quantitative differences between the GM tomato that was GM for resistance to TSWV and the non-GM control. Similarly, Di Carli et al. (2009) demonstrated that expression of scFv(G4) against the CMV coat protein in tomato did not cause pleiotropic effects. Di Carli et al. (2010) further evaluated protein profiles of the same scFv(G4)-expressed GM tomato and the wild-type after cucumber mosaic virus (CMV) infection. Proteomic data showed that proteins related to photosynthesis, photorespiration, carbon metabolism, and defense mechanism were downregulated by CMV in infected leaves, which highlighted the mechanisms of the plant–virus interactions.

WHEAT

Wheat is the second most important cereal crop in the world and constitutes a major part of the human diet (Salekdeh and Komatsu, 2007). Currently, GM wheat mainly involves improvement in protein quality. A proteomic study of GM bread wheat overexpressing a low-molecular-weight glutenin subunit (LMW-GS) revealed a series of variations, including overaccumulation of the LMW glutenin and downregulation of all other classes of storage proteins, which constituted a compensatory mechanism (Scossa et al., 2008). The proteomic analysis of two other GM durum wheat, Svevo B730 1-1, expressing the wild-type form of tobacco *rab-1*, and Ofanto B688 1-2, expressing a mutated form of *rab-1*, and their control lines (Svevo and Ofanto) revealed inconsistent results (Di Luccia et al., 2005). Tobacco *rab-1* influences the trafficking of gluten proteins through the secretory system by up- or downregulating the transport step from the endoplasmic reticulum to the Golgi apparatus. Proteomic data showed significant differences between Ofanto B688 1-2, with downregulated trafficking, and the Ofanto control but none between Svevo B730 1-1, with upregulated trafficking, and the Svevo control. The study concluded that the proteomic approach is powerful for investigating protein changes in GM crops and for understanding events involved in quality trait. Indeed, Horvath-Szanics et al. (2006) used proteomic methods to identify stress-induced proteins in herbicide-resistant GM wheat lines and found changed level of LMW seed proteins and sensitivity to drought stress in this GM wheat under drought stress.

FUTURE AND CHALLENGES

Proteomics has been used to detect unintended effects caused by genetic modification in GM crops. Results from most studies involving comparative proteomics demonstrated essentially identical protein profiles in GM crops and controls. Furthermore, genetic modification caused less variation in GM crops than the natural variability derived from conventional breeding and genotypic variation. However, some other studies demonstrated that genetic modification affected the proteomes of recipient crops. The discrepancy may be explained by differences in (1) the inserted exogenous genes, (2) the insertion sites of exogenous genes, (3) the expression levels of exogenous genes in recipient crops, (4) the specific organs used for study, (5) GM crop planting conditions, and (6) detection techniques. Therefore, proteomic evaluation of GM crops should be considered on a case-by-case basis.

However, proteomic approaches need improvement. First, homogeneity is needed in experimental quality and data handling (Lay et al., 2006). Experiments should involve as many biological repeats as possible. Statistical analysis and biological validation are also needed. These requirements are applicable to all "omics" techniques. Second, different methods should be combined, including different combinations of "omics" as well as the combination of "omics" and targeted analysis (Ricroch et al., 2011). More sensitive techniques are needed for detecting a larger range of unintended effects.

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C_4 photosynthetic machinery: insights from maize chloroplast proteomics

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C_4 plants exhibit much higher CO_2 assimilation rates than C_3 plants under certain conditions. The specialized differentiation of mesophyll cell and bundle sheath cell type chloroplasts is unique to C_4 plants and improves photosynthetic efficiency. Maize (*Zea mays*) is an important crop and model with C_4 photosynthetic machinery. 2DE and high-throughput quantitative proteomics approaches (e.g., isobaric tags for relative and absolute quantitation and shotgun proteomics) have been employed to investigate maize chloroplast structure and function. These proteomics studies have provided valuable information on C_4 chloroplast protein components, photosynthesis, and other metabolic mechanisms underlying chloroplast biogenesis, stromal, and membrane differentiation, as well as response to salinity, high/low temperature, and light stress. This review presents an overview of proteomics advances in maize chloroplast biology.

Keywords: maize, chloroplast, proteomics, C_4 plant, photosynthesis

INTRODUCTION

Chloroplasts are organelles for photosynthesis. Chloroplasts also participate in the amino acid, vitamin, isoprenoid, and lipid biosynthesis, as well as reduction of nitrite and sulfate (van Wijk, 2000; Baginsky and Gruissem, 2004). A previous study has proposed that there are ∼3000 proteins in mature chloroplasts that have specialized distributions and functions (Leister, 2003). Based on the primary product of carbon fixation, plants are classified as C_3 and C_4 species. Oxaloacetate (a four-carbon compound) and 3-phosphoglycerate (a three-carbon compound) are the primary products of carbon assimilation in the C_4 and C_3 plants, respectively. Under certain conditions, the CO_2 assimilation rate of C_4 plants is much higher than that of C_3 plants. In addition, C_4 photosynthesis enables higher nitrogen and water use efficiency than C_3 photosynthesis. Maize (*Zea mays*) is a representative C_4 plant of the nicotinamide adenine dinucleotide phosphate (NADP)-malic enzyme type. Primary carbon fixation and reduction are spatially separated between two different cell types, mesophyll cells (M) and bundle sheath cells (BS). C_4 plant M and BS have morphologically and biochemically distinct features and cooperate in photosynthesis (Edwards et al., 2001; Majeran et al., 2005). C_4 chloroplasts in M contain grana thylakoids, linear electron transport, and product reduced NADP (NADPH); while chloroplasts in BS are agranal and depleted of photosystem II (PSII), and perform most of the Calvin cycle reactions. The differentiation of C_4 chloroplasts in different cell types is regulated by a complex network at both gene (Sawers et al., 2007) and protein (Majeran et al., 2005) levels. The C_4 photosynthetic mechanism is a sophisticated signaling and metabolic network. In the past 7–8 years, high-throughput quantitative proteomics studies on the C_4 plant maize chloroplasts have been carried out. These proteomics investigations, mainly carried out by the van Wijk's lab, have been able to consolidate previous scattered information and provide new insights into the fine-tuned chloroplast biogenesis/differentiation in the M and BS, chloroplast stress response, and toward understanding C_4 photosynthetic machinery. Especially, the studies from van Wijk's lab provided several new insights into NADPH type C_4 photosynthesis and the distribution of protein functions across BS and M chloroplasts (Majeran et al., 2005, 2008; Covshoff et al., 2008; Majeran and van Wijk, 2009; Friso et al., 2010). In this mini review, we mainly aim to present a brief summary of current quantitative proteomics studies of maize chloroplasts.

C_4 CHLOROPLAST STROMAL PROTEOME IN M AND BS

Chloroplast stromal proteins from maize M and BS were identified using gel-based and gel-free proteomics approaches (Majeran et al., 2005; Friso et al., 2010). The differentially accumulated proteins from the two types of chloroplasts are mainly involved in primary metabolisms, redox regulation, gene expression, and protein homeostasis. The proteomics results yield several new insights into cellular specialization of the C_4 photosynthesis.

Cell type-specific distribution of many biochemical processes exist in the M and BS chloroplasts (**Figures 1A,B**). For instance, the reductive phase of the Calvin cycle, reversible pentose phosphate pathway (PPP), oxidative PPP, methylerythritol phosphate pathway, and amino acid metabolism (e.g., biosynthesis of arginine, branched amino acids, and aromatic amino acids) are more active in the M chloroplasts than that in BS (Majeran et al., 2005; Friso et al., 2010). Based on proteomics results, most of the enzymes involved in the above processes were preferentially expressed in the M chloroplasts, suggesting high demand for various metabolites in the M chloroplasts (Friso et al., 2010; **Figure 1A**). In contrast, most of the enzymes involved in starch metabolism were more abundant in the BS chloroplasts than in the M chloroplasts (**Figure 1B**). This is consistent with the fact that BS chloroplasts possess more starch particles. However, various proteins involved in fatty acid synthesis were equally distributed across M and BS chloroplasts (**Figure 1E**), indicating the similar demands for fatty acids in both M and BS chloroplasts (Majeran et al., 2005; Friso et al., 2010).

Reactive oxygen species (ROS) production and redox balance play important roles in regulating plastid functions (Baier and Dietz, 2005). Proteomics results revealed that the majority of ROS scavenging enzymes showed high abundance in the M chloroplasts (**Figure 1A**). This is proposed to be associated with high linear electron transport rate and water-splitting activity of PSII in the M chloroplasts (Majeran et al., 2005; Majeran and van Wijk, 2009). In addition, a great portion of nucleotide metabolism-related enzymes, such as adenylate monophosphate kinase 2, nucleoside diphosphate kinase 2, soluble inorganic pyrophosphatase, and membrane-bound adenosine triphosphate (ATP)/adenosine diphosphate (ADP) translocator, showed preferential accumulation in the M chloroplasts (Friso et al., 2010; **Figure 1A**). Since *de novo* biosynthesis of nucleotides is energy consuming, the M chloroplasts could generate adequate energy through linear and cyclic electron transport (Zrenner et al., 2006).

The components of M and BS chloroplast protein synthesis machineries show overlapping but different expression patterns

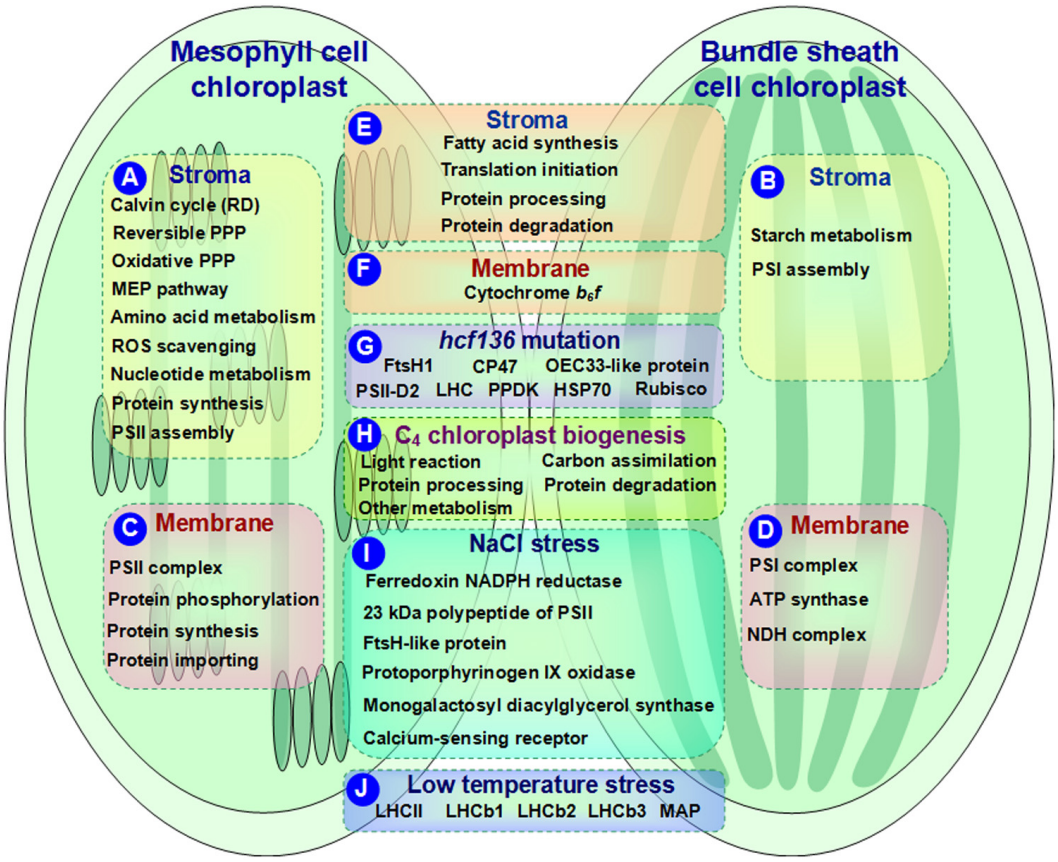

FIGURE 1 | Schematic presentation of cell-specific or stress-responsive pathways and proteins in maize chloroplasts revealed from proteomics studies. (A,B) Preferential metabolic pathways in the stroma of M and BS, respectively; **(C,D)** Preferential metabolic pathways and protein complexes in the chloroplast membrane of M and BS, respectively; **(E,F)** Metabolic pathways and protein complexes equally distributed in the chloroplast stroma/membrane of M and BS; **(G)** Differentially expressed proteins in chloroplasts of maize *hcf136* mutant in comparison to wild-type; **(H)** Dynamics of metabolic pathways during maize chloroplast biogenesis; **(I)** Salt-responsive proteins in maize chloroplasts; **(J)** Low temperature-responsive proteins in maize chloroplasts. ATP, adenosine triphosphate; HSP, heat shock protein; LHC, light harvesting complex; MAP, minor antenna proteins; MEP, methylerythritol phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NDH, NAD(P)H dehydrogenase; OEC, oxygen evolving center; PPDK, pyruvate orthophosphate dikinase; PPP, pentose phosphate pathway; PSI, photosystem I; PSII, photosystem II; RD, reductive phase; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

in C_4 plants. Comparative proteomics analysis showed that the majority of initiation and elongation factors (involved in protein translation initiation), general chaperones (related to protein processing), and Clp proteases (participate in protein degradation) were equally distributed across M and BS chloroplasts (**Figure 1E**). In contrast, ribosomal proteins and tRNA synthases, involved in protein synthesis, were much higher in the M chloroplasts than in the BS (**Figure 1A**). This implies that in the M chloroplasts, there is more protein synthesis which is required for repairing the chloroplast-encoded reaction center protein D1 (Baena-Gonzalez and Aro, 2002). Additionally, assembly factors for PSII complexes showed higher abundance in the M chloroplasts (**Figure 1A**), while photosystem I (PSI) complex assembly factors were preferentially expressed in the BS chloroplasts (**Figure 1B**). The well-correlated expression of proteins in the M and BS chloroplasts suggests existence of well-developed regulatory networks in C_4 photosynthesis (Friso et al., 2010).

C_4 CHLOROPLAST MEMBRANE PROTEOME IN M AND BS

Maize thylakoid membrane proteins play key roles in C_4 photosynthesis. Thirty-four thylakoid membrane proteins were identified and quantified using shotgun proteomics approaches (Liu et al., 2011). The majority of the proteins (∼76%) were involved in photosynthetic light reactions. Among them, only two PSI subunits were detected, suggesting that most of the PSI components accumulated at lower levels. In addition, a comparative proteomics study on the M chloroplast envelopes between maize and C_3 plant pea (*Pisum sativum*) revealed that C_4- and C_3-type chloroplasts contained qualitatively similar but quantitatively different membrane protein components (Brautigam et al., 2008). For instance, several translocators (e.g., outer envelope porin, triosephosphate translocator, and phospho*enol*pyruvate translocator) showed higher abundance in C_4 chloroplast envelopes than in C_3 plants. However, two protein import complex components, Tic55 and ClpC/Hsp93, were found at lower expressional levels in C_4 chloroplast envelopes (Brautigam et al., 2008). These data imply that the C_4 chloroplast envelope transporters are adapted to meet the demand of high metabolic flux rates during C_4 photosynthesis. However, the small number of proteins identified in these studies provides limited information toward understanding the dynamics and functions of C_4 thylakoid membrane proteins.

Current quantitative proteomics approaches (e.g., isobaric tags for relative and absolute quantitation (iTRAQ) and label-free quantification) provide more information for understanding the differentiation and oligomeric states of membrane proteins in the C_4 chloroplasts of BS and M (Majeran et al., 2008; Friso et al., 2010). For instance, the contents of PSI and PSII complexes were more abundant in the BS (**Figure 1D**) and M (**Figure 1C**) thylakoids, respectively. This is consistent with their specific roles in the corresponding cell types. Besides, ATP synthase was increased in the BS thylakoids (**Figure 1D**), while cytochrome b_6f was unchanged between the two cell types (**Figure 1F**). In addition, NAD(P)H dehydrogenase (NDH) complex showed a preferential BS accumulation (Ivanov et al., 2007; **Figure 1D**). NDH is involved in chlororespiration and cyclic electron flow around PSI. A novel subcomplex of NDH complex was also identified in the BS thylakoids (Majeran et al., 2008), which is speculated to be responsible for carbon concentrating especially when ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate is lower than the malic enzyme decarboxylation rate.

Proteomics analysis revealed that different paralogs with cell-specific accumulation patterns existed in the M and BS chloroplast membranes. For example, two light harvesting complex II (LHCII) members exhibited higher BS/M ratios than other LHCII proteins (Majeran et al., 2008). Additionally, the differential BS/M ratios of PSI subunits paralogs might reflect the distinct PSI composition between the BS and M thylakoids (Majeran et al., 2008). Further studies assisted by high quality annotated maize genome sequences are needed for exploring the subtle but interesting differences.

The ROS scavenging system and (de)phosphorylation-driven protein state transitions have been employed by C_4 plants during the M and BS differentiation to avoid light stress and optimize light harvesting capacity. Proteomics results showed that the low BS/M ratio for lumenal peroxidase-Q but high BS/M ratio for peroxidase-E implied the distinct ROS scavenging mechanisms in the M and BS chloroplasts. Several light stress protein homologs exhibited preferential accumulation in the M chloroplasts (Majeran et al., 2008). Furthermore, thylakoid kinases, such as state transition kinase (STN) 7, STN8, phosphoprotein TSP9, and lumenal isomerase TLP40, exhibited low BS/M ratios (Majeran et al., 2008; **Figure 1C**). These enzymes may be involved in (de)phosphorylation-driven protein state transitions in the M thylakoids to balance the excitation of PSI and PSII reaction centers.

To maintain protein homeostasis within different cell types, C_4 plants differentiated distinct machineries for protein synthesis, assembling, importing, processing, and degradation in the M and BS chloroplasts. The differentially accumulated proteins in maize chloroplast membranes of the M and BS support the following metabolic mechanisms: (1) The preferential M accumulation of ribosomal proteins suggest high translation rates in the M chloroplasts, which can contribute to the high abundance of PSII subunits and short lifetime of PSII reaction center proteins caused by light-induced damage (Friso et al., 2010; **Figure 1C**). (2) In terms of protein assembly, the preferential accumulation of low PSII accumulation 1 protein (LPA1) in the M chloroplasts is consistent with its role in D1 protein synthesis (Peng et al., 2006). However, it was intriguing that high chlorophyll fluorescence 136 (HCF136) responsible for assembling PSII reaction centers (Covshoff et al., 2008) was equally distributed in the BS and M chloroplast membranes. Its specific functions in the two cell types remains to be determined (Majeran et al., 2008). (3) Highly accumulated protein importing-related proteins (e.g., Tic110, Tic21, and Tic40) in the M chloroplasts indicate an increased protein flux in the M chloroplasts (Majeran et al., 2008; **Figure 1C**). (4) As for protein degradation, lumenal DegP1 was enriched in the M thylakoids and this is consistent with its function in D1 protein degradation (Sun et al., 2007). Several proteolysis-related proteins showed distinct BS/M ratios, indicating their specific roles in the BS and M chloroplasts (Majeran et al., 2008). The role of proteolysis in the BS

and M differentiation needs to be further explored by studying the cell type-specific accumulation of proteases during BS/M development.

Key gene mutations of the M and BS chloroplasts in C_4 plants provide valuable information toward understanding the mechanisms underlying the C_4 photosynthetic machinery. Covshoff et al. (2008) identified an *Activator*-induced maize mutant that lacks PSII activity. This mutated gene is a homolog of *HCF136*, which is responsible for PSII assembly or stability (Plucken et al., 2002). *hcf136* mutant seedlings contained smaller chloroplasts in both M and BS and abnormal/no grana in the M plastids (Covshoff et al., 2008). Furthermore, PSII reaction center functionality was undetected ($F_v/F_m = 0$) in mutant plants. Consistently, PSII reaction center and core subunits were absent from the *hcf136* thylakoid membranes, while PSI was not affected (Covshoff et al., 2008). Moreover, major LHCII in the mutant seedlings displayed a monomeric form instead of the typical trimeric form of wild-type thylakoids (Covshoff et al., 2008). Additionally, the *psbB-psbH-psbT-petB-petD* polycistron, encoding the components of PSII (e.g., *psbB*, *psbH*, *psbN*, and *psbT*) and cytochrome b_6f (e.g., *petB* and *petD*), was misprocessed in the *hcf136* mutant M (Covshoff et al., 2008). These results prove that the mutation of *hcf136* leads to disruption of PSII assembly or stability. Proteomics analysis found that *hcf136* mutation led to differential accumulation of several proteins in the thylakoid membranes. These proteins were identified as FtsH1, CP47, oxygen evolving center 33-like protein, PSII-D2, LHC, pyruvate orthophosphate dikinase, heat shock protein 70 (HSP70), and Rubisco small subunit (Covshoff et al., 2008; **Figure 1G**). However, the relative levels of the gene transcripts did not correlate with corresponding protein levels. This inconsistency between transcript accumulation and protein abundance suggests the involvement of transcriptional/translational regulations during C_4 differentiation.

CHANGES IN PROTEIN ABUNDANCE DURING C_4 CHLOROPLAST BIOGENESIS

Maize greening is accompanied by the differentiation of the M and BS chloroplasts for C_4 photosynthesis. This process has long been considered as a model system to study the sophisticated mechanisms of chloroplast biosynthesis. A large-scale proteomics analysis of the leaf and the BSs with their vascular bundle along the leaf developmental gradient has provided detailed dynamic information of more than 4300 proteins for a systems-level understanding of maize leaf formation and differentiation (Majeran et al., 2010). The changes of protein expression patterns highlighted the active transition and/or differentiation of C_4 malate-pyruvate shuttle, photosynthetic linear and cyclic electron flow, photorespiration, protein translation, specific transporters, and other metabolic processes along the leaf developmental gradient (Majeran et al., 2010). Hierarchical clustering of protein expression data revealed obvious spatial differentiation characteristics. The chloroplast biogenesis-related proteins accumulated to significant levels in the first 4 cm from the leaf base, and the majority of the photosynthetic apparatus-related proteins started to accumulate significantly beyond the 4 cm from ligule. This indicates that the establishment of basic chloroplast functions takes place prior to the specific cell-type differentiation related to C_4 functions (Majeran et al., 2010).

In addition to leaf proteomics, a chloroplast proteomics study also revealed protein changes during maize greening (0∼48 h) (Lonosky et al., 2004; **Figure 1H**). (1) Proteins involved in light reactions changed during greening. For instance, ATPase is the most abundant protein identified in maize chloroplasts during greening. In general, the four subunits of ATPase increased continually with greening (0∼48 h). However, some α and β subunits showed different expression patterns possibly due to the demands for different protein forms during specific chloroplast differentiation periods. (2) In general, photosynthetic carbon assimilation-related enzymes were increased during the early time (0∼4 h) of greening. This is consistent with a previous notion that the plastid assembles the photosynthetic apparatus during early development. Afterward, some enzymes such as β-amylase, NADP malate dehydrogenase, and phosphoglycerate kinase (PGK) reached a plateau. Interestingly, two enzymes displayed opposite expression patterns. The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) kept increasing, while isoamylase began to decrease after the early phase. Moreover, the expression patterns of PGK and GAPDH were consistent with their mRNA levels reported in previous studies (Dewdney et al., 1993; Bringloe et al., 1996). (3) The plastid chaperonins and proteases also changed during maize greening. They are involved in protein processing and degradation, respectively. For instance, α subunit of the 60-kD and 20-kD chaperonins displayed moderate increases in the early (0∼4 h) and middle (12 h) phases but decreased at 48 h. In addition, both HSP70 and ClpC increased during the initial phase of greening and reached a plateau at 48 h. These data imply that the active protein folding and degradation takes place, which contribute to active alterations of protein activity and turnover in various signaling and metabolic changes during chloroplast biogenesis. (4) Proteins involved in various plastid metabolic processes (e.g., acetyl-coA carboxylase, beta-D-glucosidase, nucleic acid-binding protein) showed complex patterns of protein abundances during greening. All the above findings have provided valuable insights into the mechanisms underlying chloroplast biogenesis.

STRESS-RESPONSIVE PROTEINS IN C_4 CHLOROPLASTS

Salinity is thought to have a strong influence on plant chloroplast protein composition. Several salt-responsive proteins have been identified in maize chloroplasts undergoing 25 mM NaCl treatment for 4 h using 2DE-based proteomics approaches (Zorb et al., 2009; **Figure 1I**). In the salt-stressed maize plants, three photosynthesis-related proteins (i.e., ferredoxin NADPH reductase, 23 kDa polypeptide of PSII, and FtsH-like protein) were increased under NaCl stress. This would help to attenuate the severe effects of Na^+ on the photosynthetic machinery. Additionally, the enhanced abundance of protoporphyrinogen IX oxidase was detected in salt-treated maize chloroplasts. This enzyme is involved in heme and chlorophyll biosynthesis, and its substrates are the targets of salt toxicity leading to massive oxidative stress. The increment of protoporphyrinogen IX oxidase would contribute to alleviate oxidative stress in salt-stressed maize

chloroplasts. Monogalactosyl diacylglycerol synthase and calcium-sensing receptor were salt-reduced in the maize chloroplasts. These two enzymes are involved in membrane maintenance and Na^+ sensing, respectively. These results provide valuable information for future studies on the molecular mechanisms of salt tolerance in maize chloroplasts.

High/low temperature and light effects on maize photosynthetic apparatus have been investigated (Caffarri et al., 2005). Low temperature led to decreases of chlorophyll contents and F_v/F_m in maize indicating that low temperature could cause photoinhibitory damage to the PSII reaction center. In addition, maize plants grown under low light and high temperature conditions exhibited an increased value of non-photochemical quenching. Under multiple temperature and light conditions, low temperature is the principal factor that affects protein expression in maize thylakoid membranes. For instance, LHCII contents in maize plants under low temperature were higher than under high temperature. Minor antenna proteins were decreased compared to the LHCII proteins in maize plants grown under low temperature (**Figure 1J**). In addition, nine LHCb1, two LHCb2, and three LHCb3 protein spots were positively detected by corresponding antibodies on 2DE gels (Caffarri et al., 2005; **Figure 1J**). They presented diverse expression patterns under different temperature/light conditions. This suggests that different genes were translated into proteins of thylakoid membranes in response to environmental stress, which might be a basic mechanism in the C_4 photosynthetic apparatus for environmental adaptation.

CONCLUSION

The maize chloroplast is a good model for studying the C_4 photosynthetic mechanism. The development of large-scale quantitative proteomics approaches together with the availability of maize genome sequences has provided a high-throughput platform with high resolution and sensitivity for analyzing protein expression patterns in the M and BS chloroplasts of maize. The quantitative proteomics information acquired to date provides new insights into the specific C_4 chloroplast biogenesis, M and BS differentiation, and stress response. However, the photosynthetic machinery and metabolic mechanisms are too complicated to be interpreted by just using quantitative protein profiles. Specialized protein complexes, protein-protein interaction, and post-translational modifications have been proposed to play key roles in photosynthesis. Thus, further proteomics studies should focus on the analysis of large-scale protein modifications and interactions to enhance our understanding of the protein networks in C_4 photosynthesis.

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Comparative proteomics of chloroplasts envelopes from bundle sheath and mesophyll chloroplasts reveals novel membrane proteins with a possible role in C4-related metabolite fluxes and development

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As the world population grows, our need for food increases drastically. Limited amounts of arable land lead to a competition between food and fuel crops, while changes in the global climate may impact future crop yields. Thus, a second "green revolution" will need a better understanding of the processes essential for plant growth and development. One approach toward the solution of this problem is to better understand regulatory and transport processes in C4 plants. C4 plants display an up to 10-fold higher apparent CO_2 assimilation and higher yields while maintaining high water use efficiency. This requires differential regulation of mesophyll (M) and bundle sheath (BS) chloroplast development as well as higher metabolic fluxes of photosynthetic intermediates between cells and particularly across chloroplast envelopes. While previous analyses of overall chloroplast membranes have yielded significant insight, our comparative proteomics approach using enriched BS and M chloroplast envelopes of *Zea mays* allowed us to identify 37 proteins of unknown function that have not been seen in these earlier studies. We identified 280 proteins, 84% of which are known/predicted to be present in chloroplasts. Seventy-four percent have a known or predicted membrane association. Twenty-one membrane proteins were 2–15 times more abundant in BS cells, while 36 of the proteins were more abundant in M chloroplast envelopes. These proteins could represent additional candidates of proteins essential for development or metabolite transport processes in C4 plants. RT-PCR confirmed differential expression of 13 candidate genes. Chloroplast association for seven proteins was confirmed using YFP/GFP labeling. Gene expression of four putative transporters was examined throughout the leaf and during the greening of leaves. Genes for a PIC-like protein and an ER-AP-like protein show an early transient increase in gene expression during the transition to light. In addition, *PIC* gene expression is increased in the immature part of the leaf and was lower in the fully developed parts of the leaf, suggesting a need for/incorporation of the protein during chloroplast development.

Keywords: C4 plant, chloroplast envelope proteins, photosynthesis, mesophyll cells, bundle sheath cells

INTRODUCTION

Changes in the world population have drastically increased our need for food and fuel. When faced with similar issues in the 1940s, the "green revolution," led by Norman Borlaug, involved the development of high-yielding varieties of cereal grains, modernization of management techniques and irrigation systems, as well as distribution of hybridized seeds, synthetic fertilizers, and pesticides to farmers. Today, the amount of arable land is limited and often there is now a competition between food and fuel crops. In addition, changes in the global climate may impact future yields. To continue to be able to provide sufficient food and fuel, we need plants that show accelerated growth, have a higher grain, or cell wall yield/quality, and are more resistant to biotic and abiotic stressors.

One adaptation of plants in response to a dry environment is C4 photosynthesis. This process allows for biomass accumulation with high nitrogen and water use efficiency (Leegood and Edwards, 1996; Sage, 2004), a trait which increases the productivity of crop plants (Matsuoka et al., 1998). During photosynthesis in the C4 plant *Zea mays*, primary CO_2 fixation and the subsequent carbon reduction are spatially separated into mesophyll (M) and bundle sheath (BS) cells, respectively. Maize belongs to the NADP-malic enzyme type of C4 plants (Hatch, 1987). In maize mesophyll cells, CO_2 is initially fixed *via* phosphoenolpyruvate (PEP) – carboxylase to oxaloacetate (OAA), transported into the chloroplast, and converted to malate by NADP-malic enzyme (ME). Malate moves from the surrounding mesophyll cells into BS cells and

is decarboxylated in the chloroplast, yielding CO_2, NADPH, and pyruvate. CO_2 and NADPH enter the Calvin–Benson Cycle where CO_2 is reduced to triose phosphates, while pyruvate is transported back to mesophyll cells, imported into chloroplasts, and converted back to the primary CO_2 acceptor PEP by the enzyme phosphoenolpyruvate phosphate dikinase (PPDK). In addition to the enrichment of CO_2 around Rubisco, the oxygenation reaction of the enzyme is further reduced by a limited PSII reaction and thus reduced O_2 production in the BS chloroplast (Meierhoff and Westhoff, 1993). These processes require the shuttling of intermediates as well as reduction equivalents between cells and organelles and consequently across several membranes. As a result, chloroplasts of mesophyll and BS cells have adapted to their respective roles (Slack et al., 1969; Edwards et al., 2001; Majeran et al., 2005) and are functionally different from each other as well as from chloroplasts in C3 plants (Bräutigam et al., 2008). Despite detailed knowledge about the soluble proteins involved in and necessary for C4 photosynthesis and an increasing body of information about the chloroplast membrane proteome in both C3 and C4 plants (Bräutigam et al., 2008; Majeran et al., 2008), many aspects of the adaptation of integral and peripheral membrane proteins as well as the necessary regulatory proteins remain unknown. Here, we focus on analyzing the quantitative and qualitative differences between isolated chloroplast envelope membranes of BS and mesophyll cells, followed by localization and expression studies to further understand the possible impact of newly described envelope proteins.

Two membranes separate the chloroplast from the remainder of the plant cell: the outer and the inner envelope. Metabolite transport through the outer envelope is largely controlled through substrate-specific pore proteins (Pohlmeyer et al., 1997, 1998; Bolter et al., 1999; Goetze et al., 2006), while transport across the inner envelope is mediated by a large number of specific transporters (Weber, 2004; Weber et al., 2005; Weber and Fischer, 2007). The spatial separation between primary CO_2 fixation and carbon reduction and the resulting necessary movement of metabolites, requires at least four transport processes. Good candidates for PEP export, triosephosphate shuttling, and oxaloacetate/malate transport have already been described: three maize homologs of the inner envelope DIT (dicarboxylate transporter), DIT1, and DIT2, likely function as 2-oxoglutarate/malate translocator and are expressed in the mesophyll envelopes and BS envelopes, respectively (Taniguchi et al., 2004; Majeran et al., 2005). The function of the third DIT homolog, also named 2-oxoglutarate/malate transporter 1 (OMT1), remains unclear (Taniguchi et al., 2004). Other putative mesophyll envelope transporters, mesophyll envelope proteins MEP 1–4, were expressed in both mesophyll and BS, whereas MEP3 in the BS (Majeran et al., 2005; Bräutigam et al., 2008). The molecular nature of others, for example the predicted pyruvate transporter, is unknown. Likewise, it is unknown whether the same or different transport proteins mediate metabolite transport across the mesophyll and the BS chloroplast envelope and whether additional transporters exist. In addition, new proteins necessary for regulating the differential development of BS and mesophyll chloroplasts may form new membrane receptors or may need to be transported into the chloroplast, thus appearing in envelope proteomes.

In this work, we compared the proteome of purified envelopes of BS and mesophyll chloroplasts to identify further components of C4 metabolite transport. We hypothesized that this enrichment step will allow us to identify differentially distributed yet less abundant and previously undescribed integral or peripheral membrane proteins as well as putative regulatory proteins imported into the chloroplast. We applied a direct quantification method, the total spectral count of proteins (number of mass spectra that map to one protein), which has been used to analyze large datasets of proteins, to compare the relative abundance of BS and mesophyll chloroplast envelope proteins (Liu et al., 2004; Zybailov et al., 2005; Lu et al., 2007; Bräutigam et al., 2008; Majeran et al., 2008). GFP labeling confirmed their localization at the chloroplast. Furthermore, we used RT-PCR to correlate the gene expression of several of the newly identified putative membrane proteins with chloroplast development and protein levels to better understand their putative function.

MATERIALS AND METHODS

PLANT MATERIAL

Zea mays Great Lakes 4758 hybrid seeds were rinsed thoroughly to remove fungicides and shaken in water for up to 1 h to speed germination. Kernels were planted in a standard soil mixture containing equal parts of Bacto Soil (Michigan Pear Company, Houston), medium vermiculite, and perlite. Plants were grown either in complete dark for extraction of mRNA associated with development or at a 12 h day/12 h night cycle at a daytime temperature of 22°C for BS-mesophyll comparisons. For expression studies of chloroplast envelope proteins and for envelope protein preparations, plants were harvested after 6 weeks. For the light-induction experiment, plants were kept in the dark for the first 6 days of germination and then transferred to light.

ISOLATION OF MESOPHYLL PROTOPLAST AND BS STRANDS

Leaves of 4–6-week-old corn plants were collected and the midrib removed. Mesophyll protoplast and BS strands were isolated following the method by Kanai and Edwards (1973) with some modifications. In short, 5 g of leaves were cut into thin slices (0.5–0.7 mm wide). Leaf slices were incubated in100 ml of digestion media (0.6 M Sorbitol; 20 mM MES (pH 5.5); 5 mM $MgCl_2$, and 2% Cellulase (PhytoTechnology Laboratories, Overland Park, KS, USA). The flask was put under vacuum for 5 min, followed by incubation in a shaker (60 rpm) at 30°C for 2 h. The digestion media was discarded, 100 ml of fresh media added to the leaves, and the incubation repeated for an additional hour. Digestion media was discarded and 30 ml of 0.6 M Sorbitol was added, followed by gentle shaking for 15 min. The wash was filtered first through a tea strainer, then through 80 μm nylon filter. This process was repeated twice and the washes were centrifuged at $300 \times g$ for 3 min. The supernatant was discarded and the pellet was further purified using the two phase system as described (Kanai and Edwards, 1973) to obtain pure mesophyll protoplast. The purified protoplast was stored at −80°C. BS strands collected on the 75 μm mesh was washed with Sorbitol medium [0.6 M Sorbitol, 0.05 M Tricine-KOH (pH 8.0); 5 mM $MgCl_2$], mixed with a vortex for 10 s, and filtered through 75 μm nylon filter. BS strands were collected from the nylon filter and stored at −80°C.

PREPARATION OF CHLOROPLAST ENVELOPES

Purified, intact chloroplast were broken in rupture buffer (10 mM Tricine/KOH pH 7.5, 1 mM PMSF, 5 mM EDTA), layered over 21% sucrose and 45% sucrose in TE, and ultra-centrifuged at 180000 × *g* for 90 min (Cline et al., 1981). The yellow band was recovered as chloroplast envelopes were collected, while the precipitate was recovered as crude chloroplast fraction. Envelope membranes were diluted with TE containing 1 mM PMSF, pelleted by centrifugation for 1 h at 25 g, resuspended in TE/PMSF, and stored at −80˚C.

PROTEIN IDENTIFICATION

For proteomics analysis, mesophyll and BS envelope membranes from two and three individual preparations, respectively, were dissolved in sample buffer and separated using 10% SDS–PAGE. After staining, each gel lane was cut into 10 equally sized slices. Gel slices were subjected to tryptic digest as described by Shevchenko et al. (1996) and analyzed according to Bräutigam et al. (2008). In short, peptides were loaded onto a Waters Symmetry C18 peptide trap (5 μm, 180 μm × 20 mm) at a flow rate of 4 μL/min in 2% acetonitrile/0.1% formic acid for 5 min. The peptides were separated on a Waters BEH C18 nanoAcquity column (1.7 μm, 100 μm × 100 mm) using a Waters nanoAcquity UPLC coupled to a ThermoElectron LTQ-FTICR mass spectrometer (flow rate of 300 nl/min; buffer A = 99.9% water/0.1% formic acid, buffer B = 99.9% acetonitrile/0.1% formic acid: gradient of 5% B to 40% B from 0 to 63 min, 40% B to 90% B from 63 to 71 min, and 5% B from 71 to 90 min). Survey scans were taken at a resolution of 50000 and the top 10 ions were subjected to automatic low-energy CID. The BioWorks Browser version v3.2 converted the resulting MS/MS spectra to a peak list.

DATA ANALYSIS

Scaffold[1] was used to validate MS/MS-based peptide and protein identifications using the Peptide Prophet algorithm (Keller et al., 2002). Parameters were set at 95% confidence for protein identification requiring at least two unique peptides for each protein, and 95% confidence for all peptides counted (shown in Table S2 in Supplementary Material). Where Scaffold reported multiple proteins identified for the same peptides, each match was manually inspected and low-scoring matches were discarded. Proteins were compared to sequence databases *Zea mays*[2]. Individual matching of tryptic fragments to predicted proteins was confirmed manually. Identified proteins were imported into Microsoft Excel for further analyses.

Each sequence was compared with the Arabidopsis proteome using blastx in plprot (Altschul et al., 1997) in TAIR and the *Arabidopsis* gene identifier (AGI) of the closest homolog was recorded. Proteins were then searched against PPDB[3]. Targeting prediction and membrane-spanning regions were achieved by using the software programs TargetP (Emanuelsson et al., 2000), ChloroP (Emanuelsson et al., 1999), WoLFPSORT (Horton et al., 2006), and Octopus (Viklund and Elofsson, 2008).

[1] www.proteomesoftware.com

[2] ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Zea_mays

[3] http://ppdb.tc.cornell.edu

SEMIQUANTITATIVE ANALYSIS OF PROTEIN ABUNDANCE

The semiquantitative analysis of protein abundance was based on the spectral count (i.e., the number of mass spectra mapping to a given protein in a single experiment) and performed according to Bräutigam et al. (2008). In short, all proteins in the sample were separated by SDS-PAGE and identified by liquid chromatography-electrospray ionization-MS/MS without prior fractionation ("whole envelopes"). The spectral counts for each protein were summed to yield the "sum" fraction. For all five data sets, spectral counts for each protein were normalized to the total number of spectra within the experiment ("percentage of the total spectral count"; Table S3 in Supplementary Material).

RNA ISOLATION AND RT-PCR

Total RNA was isolated from the mesophyll protoplast and BS strands using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using SuperscriptIII Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RNA concentration and quality were determined with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For PCR, GoTaqGreen master mix (Promega, Madison, WI, USA) or Failsafe PCR buffers (Epicenter, Madison, WI, USA) were used. For each primer set, the optimum amount of cDNA for the PCR reaction was determined by testing a series of cDNA dilutions with a fixed number of PCR cycles.

Gene-specific PCR primers were used (Table S1 in Supplementary Material) for analyzing the abundance of the transcripts of the individual genes in mesophyll and BS samples. 18S was used as an internal control. To check for the purity of the sample, PEPC and Rubisco primers were used as markers for mesophyll and BS protoplast, respectively. PCR products were visualized by agarose gel electrophoresis and the gel image was taken using a gel documentation system (Fotodyne Inc., Hartland, WI, USA). The intensity of the bands was quantified using ImageQuant software version 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). The expression levels of the individual gene in mesophyll and BS samples were compared after normalization to 18S. RT-PCR was repeated using three to five different sets of mesophyll protoplast and BS strands.

SUBCELLULAR LOCALIZATION

Coding sequence for the *Arabidopsis* homologs of ERaP, 5-TM, Mep 3, UP-a, UP-d, Hyp g, and PIC were amplified using the gene-specific primers and the PCR products were cloned into pDONR207 by BP recombination reaction, sequenced, and subcloned into pEarleyGate103 vector by LR recombination reaction to generate the expression constructs (Earley et al., 2006). The constructs were transformed into *Agrobacterium tumefaciens* C58C1pGV2260 and the transformant cultures were used for infiltrating *Nicotiana tabacum* leaves. Expression of the GFP fusion proteins were analyzed by confocal microscopy (Carl Zeiss, USA).

EXPRESSION STUDIES OF CHLOROPLAST ENVELOPE PROTEINS

Roots, mesocotyl, and three sections of corn leaves were harvested and frozen in liquid nitrogen. For the leaf samples, we used a 1 cm section still inside the leaf sheath (IL), which was etiolated and is a sink tissue. Leaf sample 2 (ML), was taken from the middle of the leaf and corresponds to "part 5" of the leaf as described by

Pick et al. (2011). Cells in this part of the leaf have been shown to contain developed chloroplasts and are a source tissue, however, they may still be expanding. Leaf sample 3 (LT), corresponds to "part1/2" described by Pick et al. (2011); it is a source tissue with fully developed cells. The samples were ground to a fine powder and RNA was extracted using a commercial RNA extraction kit provided by Qiagen. RNA concentrations were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For cDNA synthesis, 600 ng mRNA per sample was reverse transcribed using SuperscriptIII Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR primers used for the respective genes are listed in Table S1 in Supplementary Material. Identity of the PCR products was confirmed by size and sequencing. The intensity of the bands was determined as described above and normalized to 18S. The mean and standard error was calculated from three biological replicates. Three different sets of experiments were performed: (I) BS/MS comparison, (II) distribution of the gene expression in 6-week-old plants and (III) expression changes during the development of the chloroplast. For the latter, 6-day-old dark-grown corn plants were transferred to continuous light and gene expression was studied after transition to light. RNA was extracted from primary leaves every 2 h from the start of light exposure and expression of three to six biological replicates examined as described above.

RESULTS AND DISCUSSION

IDENTIFICATION AND RELATIVE QUANTIFICATION OF PROTEINS ASSOCIATED WITH THE ENVELOPE OF BS AND MESOPHYLL CHLOROPLASTS

We identified 280 proteins in our chloroplast envelope preparations from BS and mesophyll cells (Table S2 in Supplementary Material). Of these, 84% (230 proteins) were shown to be associated with chloroplasts (WoLF PSORT[4]; Horton et al., 2006; bar.utoronto.ca; Winter et al., 2007), 5% each are localized in mitochondria or cytoplasm/vacuole, respectively (**Figure 1A**). About 75% of all identified proteins are known or predicted to have membrane association, while 16% are known soluble proteins and 9% are unknown proteins without transmembrane regions (**Figure 1B**). The majority of the soluble proteins are known chloroplast stroma proteins and were likely purified during transit across the chloroplast envelope. Despite the fact that large chloroplast proteome databases already exist, we were able to detect 37 proteins of unknown or unconfirmed function that are not present in the 15 largest databases (Peltier et al., 2002; Ferro et al., 2003; Froehlich et al., 2003; Schleiff et al., 2003; Friso et al., 2004; Kleffmann et al., 2004; Peltier et al., 2004; von Zychlinski et al., 2005; Kleffmann et al., 2006; Siddique et al., 2006; Sirpiö et al., 2007; Tyra et al., 2007; Ferro et al., 2010; Weber, 2010; Breuers et al., 2011; Fischer, 2011; Marchler-Bauer et al., 2011; Kriechbaumer et al., 2012; Lundquist et al., 2012; Majeran et al., 2012). This confirms that further fractionation of the chloroplast can lead to the discovery of more novel proteins. Determining the ratio of spectral ion counts between BS and mesophyll (M) cells revealed that 67 proteins showed possible differential abundance (as confirmed by t-test, $p < 0.1$; Tables S3 and S4 in Supplementary Material). A BS/M ratio of less than 0.75 indicated mesophyll association while a ratio of larger than 1.5 suggested BS localization (**Figure 2**). The proteins identified predominantly in the mesophyll samples contain 10 subunits of different ATPases, proteins involved in photosynthetic electron transport, as well as OEE3-1 and a FtsH proteins. This is consistent with the fact that photosystem II (PSII) is down-regulated in the BS cells of C4 plants (Meierhoff and Westhoff, 1993; Majeran and van Wijk, 2009), and as a result, proteins involved in PSII should be more abundant in mesophyll cells. This includes not only proteins directly involved in PSII electron transport but also the components of the FtsH complex, which play a direct role in the maintenance of PSII (Kato et al., 2009), and oxygen evolving enhancer proteins (OEE), which are part of the oxygen evolving system of PSII.

The proteins with higher abundance in BS cells include several enzymes of the Calvin–Benson–Bassham Cycle (Fructose-bisphosphate aldolase, Sedoheptulose-1,7-bisphosphatase; Bassham et al., 1950), sugar signaling (Hexokinase; Xiao et al., 2000), and lipid metabolism (Pheophorbide oxygenase, TGD2-like protein; Roston et al., 2011). In addition, we find a stomatin-like protein (protease), several known transporters/channels (2-oxoglutarate/malate translocator: DiT/OMT; Tic110-like protein; put. ion channel protein; KEA2-like protein, voltage dependent anion channel proteins 1a, Toc159-like protein, small drug exporter, DCT2, TGD2-like protein, DiT1, ZmPIP2-3; Jarvis and Soll, 2002; Linka and Weber, 2010; Kinoshita et al., 2011; Roston et al., 2011), as well as several proteins with a possible role in electron transport (Put. NADH-ubiquinone oxidoreductase 20 kDa SU; two distinct putative NADPH/NADH dehydrogenase

[4]www.psort.org

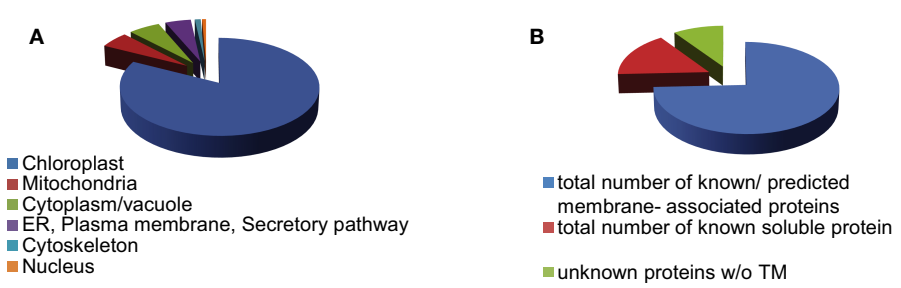

FIGURE 1 | Distribution of envelope proteins throughout different cell compartments (A) and by membrane association (B).

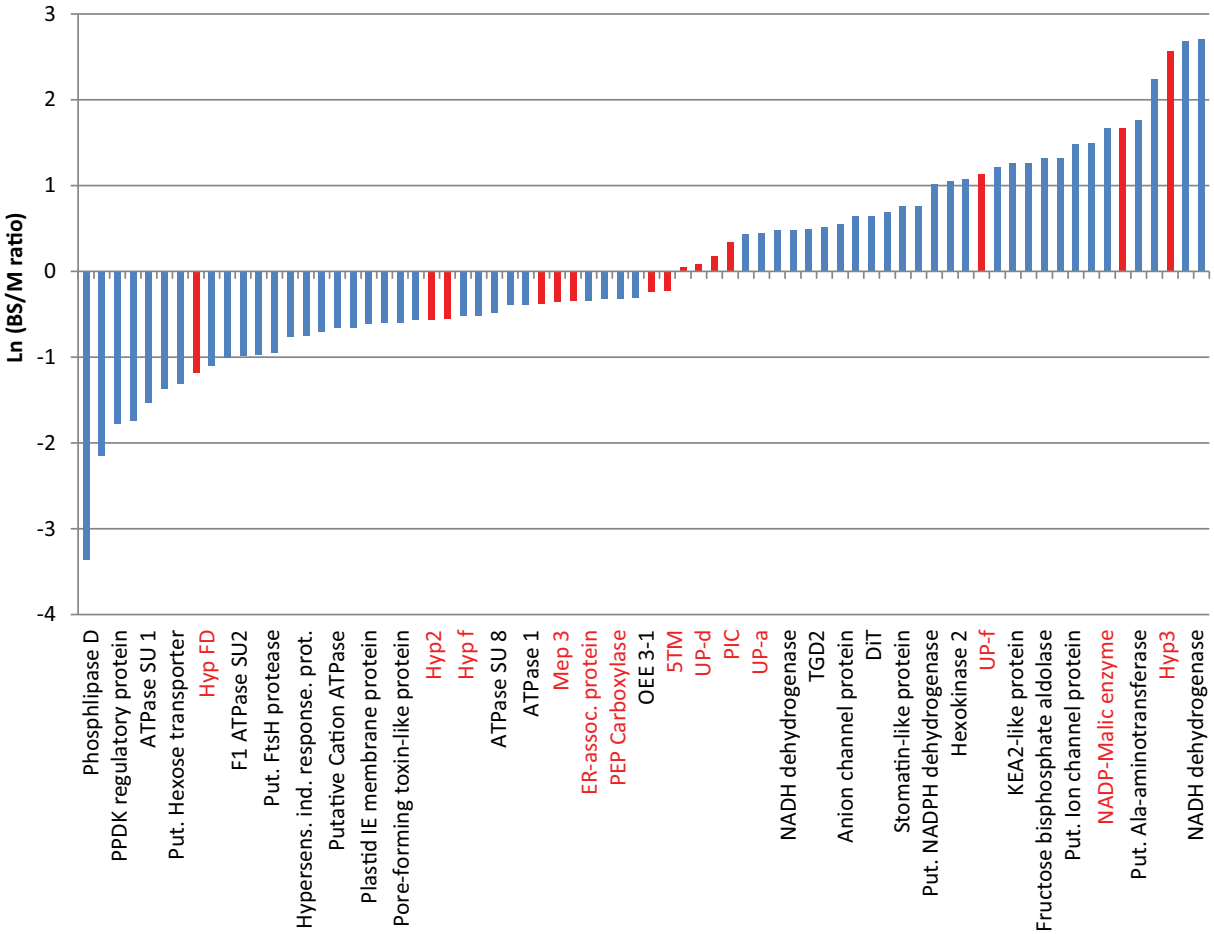

FIGURE 2 | Differences in the relative abundance of proteins based on the bundle sheath: mesophyll ratio of their spectral ion counts. Data are based on values shown in Tables S3 and S4 in Supplementary Material. Except for known controls, only membrane-associated proteins are integrated. Due to space constrains only ca. 33% of the bars are labeled. The complete protein list and figure are shown in Table S4 in Supplementary Material. Values are the averages from two mesophyll and three BS data sets. Red bars indicate proteins used for further studies.

proteins; Chlorophyll a–b binding protein 4; Rochaix, 2011). Proteins with a known or predicted role in C4 metabolism (NADP-dependent malic enzyme and a putative alanine aminotransferase; Pick et al., 2011) and eight proteins of unknown function were also found. Two of the proteins (putative NADH dehydrogenase LOC100282384, Hyp 3) were not found in the mesophyll envelope samples. Hyp3, however, was only identified in one of the samples, suggesting it is either in very low abundance or a cytoplasmic contamination. NADH-ubiquinone oxidoreductase/NADH dehydrogenases usually participate in mitochondrial electron transport, yet close relatives are found in chloroplasts. It is speculated that the chloroplast enzymes might use the quinone reductase function of the complex with a different reductant, perhaps ferredoxin or NADPH. This would corroborate their proposed function in the cyclic electron transport. It has been shown that in NADP-ME-type C4 plants the NDH complex is up-regulated in the BS, where it could contribute to the higher ATP requirement (Heber and Walker, 1992; Shikani, 2007; Rochaix, 2011). Similarly, the higher abundance of Calvin–Benson Cycle enzymes is not surprising, since carbon reduction through this path has been shown to occur in the BS cells (Taiz and Zeiger, 2006). Interestingly, one of the proteins with the largest differential abundance between BS and M cells is a putative alanine aminotransferase, which showed a BS/M ratio of 9.3. This corroborates a revised model for C4 photosynthesis in corn (Pick et al., 2011), which proposes that C4 metabolism branches after formation of oxaloacetate in the mesophyll cells. In this model both Asp and malate are formed and transported to the BS cell. Asp is converted by Asp aminotransferase (AspAT) to phosphoenolpyruvate and returned to the mesophyll cells; malate is decarboxylated to pyruvate which is either transported back to the mesophyll or converted to Ala *via* alanine aminotransferase (AlaAT). Ala then moves to the mesophyll where it is converted back to pyruvate by a second AlaAT (Pick et al., 2011). We have found both AspAT and a putative AlaAT in our samples, however only AlaAT shows a strong differential distribution with a higher abundance in the BS chloroplast (BS/M ratio: 9.3), while AspAT appears only marginally increased in the M chloroplast. Since AlaAT will likely be needed in both cell types, it is possible

that two homologs with different expression patterns may exist or that they are in compartments other than the chloroplast and would not have been detected in our dataset.

The function of several of the predicted transporters (Tic110-like, BS/MS: 3.37; KEA-2 like, BS/MS: 3.52; Put. ion channel, BS/MS: 4.38, voltage dependent anion channel proteins 1a and 2, BS/MS: 1.58/2.00, small drug exporter BS/MS:1.62, ZmPIP2-3 BS/MS:3.75) as well as that of the hypothetical proteins (Expressed protein BS/MS: 1.64, UP-f, BS/MS: 3.09; Hyp Protein/LOC100276764 BS/MS: 4.47; UP-e, BS/MS: 5.3, UP-b BS/MS: 5.86) remains to be determined.

CORRELATION OF GENE EXPRESSION WITH PROTEIN ABUNDANCE

To confirm the differential abundance of several of the chloroplast envelope proteins, we picked 13 proteins of unknown function as well as two controls (M: PEPC-phosphoenolpyruvate carboxylase; BS: NADP-ME – NADP-malic enzyme; **Table 1**) and compared their gene expression levels in BS and MS protoplast by semi-quantitative RT-PCR (**Table 2**; **Figure 3**). The proteins chosen were representatives of proteins which were more abundant in the mesophyll cells (Hyp FD, Hyp2, HypF), more abundant in the BS cells (Hyp 3, UP-f), or of equal abundance (Mep1, Mep3, ER-AP, PIC, UP-a, Up-d, 5TM, HypE). While the first and the last category may be important for M or BS-specific membrane and transport processes, the second group may be involved in chloroplast processes common to both cell types. The selected proteins were predicted to be associated with membranes or have transmembrane regions, yet their function was not well characterized (see **Table 1**):

The spectral count ratio had suggested that equal amounts of 5TM, Mep1, HypE, UP-a, UP-d, ER-AP, Mep3, and PIC/TIC are present in mesophyll and BS envelopes. Hyp2, HypF, Hyp FD are more abundant in the mesophyll cells, while Hyp3 and UP-f are slightly more abundant in the BS envelope. In most cases and in the two controls, the difference in relative amount of protein between BS and mesophyll chloroplast envelopes is closely correlated with the expression of the respective genes (within the margin of error).

Mep1 is a predicted LrgB-like protein. It is predicted to have 12 membrane-spanning regions. Mep1 (mesophyll envelope protein1) is enriched in the chloroplasts of the C4 plant maize relative

Table 1 | List of proteins used for further study and their predicted function.

Protein name	Accession no.	No. of TM regions (Octopus)	Predicted function
5TM	LOC100283913	8	*A.t.* homolog contains a DUF92 domain; predicted to be associated with the chloroplast inner envelope (NCBI).
ER-AP	LOC100283096	3	ER-associated protein but was also found in the chloroplast. Predicted to play a role in the formation of tubular ER in mammals and yeast (Nziengui et al., 2007).
Hyp2	LOC100285177	11	Contains a calcium-binding domain and may play a role in calcium modulation or signaling. Its pfam01699 domain suggests a possible role as a Sodium-Calcium exchange protein (NCBI).
Hyp3	LOC100192917	4	Protein of unknown function with a DUF3411 domain (NCBI).
HypE	LOC100275334	2	Predicted to be a member of the NADH-ubiquinone oxidoreductase complex I (NCBI).
HypF	LOC100283211	0	Similarity to chalcone isomerase (NCBI).
HypFD	LOC100282099	4	Put. PRA1-family protein. This protein family contains the glutamate transporter (EAAC1) interacting protein GTRAP3-18. Could regulate metabolite transport (Lin et al., 2001).
Mep1	LOC100383166	12	LrgB-like protein (Bräutigam et al., 2008).
Mep3	LOC100276525	4	Protein of unknown function with DUF3411 domain (Bräutigam et al., 2008; NCBI).
UP-a	LOC100285818	1	Proline-rich protein with similarity to members of the alpha-amylase inhibitors (AAI), lipid transfer (LT), and seed storage (SS) protein family (Kader, 1996, 1997; NCBI).
UP-d	LOC100192831	4	Belongs to the uncharacterized protein family, UPF0114 (NCBI).
UP-f	LOC100277914	1	Contains a MAEBL domain. MAEBL proteins were identified in *Plasmodium yoelii* and *P. falciparum* as type I transmembrane proteins with erythrocyte binding activity (Singh et al., 2004). Their function in plants is unknown.
PIC/TIC	LOC100273175	4	Contains a DUF3611. Similarity to the *A.t.* TIC21. Is predicted to be involved in copper homeostasis and protein import into chloroplasts.
PEPC	LOC100191762	0	Phosphoenolpyruvate carboxylase; initial carbon assimilation in the mesophyll cells of maize yielding oxaloacetate (Bräutigam et al., 2008).
NADP-ME	NP_001105313	2	NADP-Malic enzyme; reduction of malate in the BS chloroplast yielding CO_2, NADPH and pyruvate (Bräutigam et al., 2008).

The abbreviations for proteins are identical to those used in other tables and in the figures. Number of transmembrane regions was determined as described in Section "Materials and Methods."

Table 2 | Summary table showing membrane-associated proteins and their predicted or tested localization and bundle sheath (BS) or mesophyll (M) association.

Protein name	Localization (GFP/YFP or pred.)	No. Of TM regions (Octopus)	Spectral count ratio (BS/M)	BS/M ratio determined by RT-PCR
5TM	YFP	8	0.79	0.58 ± 0.11
ER-AP	GFP	3	0.70	0.65 ± 0.02
Hyp2	unknown	11	0.57	0.70 ± 0.27
Hyp3	Cp-pred	4	BS	0.87 ± 0.25
HypE	Cp-pred	2	0.80	0.78 ± 0.14
HypF	Cp-pred	0	0.57	0.65 ± 0.23
HypFD	Cp-pred	4	0.31	0.28 ± 0.15
Mep1	Cp-GFP	12	1.09	1.12 ± 0.56
Mep3	Cp-YFP	4	0.69	3.00 ± 1.56
UP-a	Cp-YFP	1	1.41	0.66 ± 0.11
UP-d	Cp-YFP	4	1.05	0.40 ± 0.05
UP-f	CP-pred	1	3.09	0.84 ± 0.09
PIC/TIC	Cp-YFP	4	1.20	0.99 ± 0.16
PEPC	Cyt	0	0.71	0.30 ± 0.20
NADP-ME	Cp	2	5.32	4.44 ± 1.57

*Localization prediction was based on computer prediction or on actual GFP/YFP labeling (**Figure 4**). The spectral count ratio was taken from the proteomics data (Table S2 in Supplementary Material; **Figure 2**); gene expression ratios were obtained by RT-PCR (**Figure 3**). The abbreviations for proteins are identical to those used in both supplementary tables. Spectral count ratios are based on the average of three experiments; RT-PCR-based ratios are mean and standard error of three experiments.*

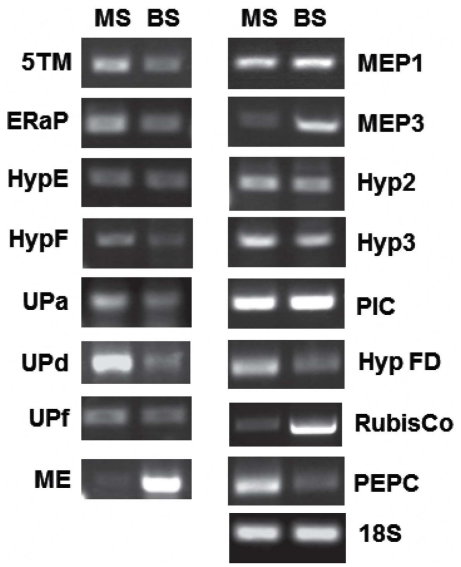

FIGURE 3 | RT-PCR showing relative abundance of transcripts for several genes encoding chloroplast envelope proteins. Band intensities of three to five biological replicates were quantified and are displayed in **Table 2**. MS, mesophyll; BS, bundle sheath.

to the C3 plant pea, but its gene expression is evenly distributed between BS and M cells in corn (Bräutigam et al., 2008). Based on our spectral count ratio, this is also true for the protein level (see **Table 2**).

ER-AP showed marginal differences in abundance between BS and M cells that were shown to be not significant using a student's *t*-test (see Table S3 and S4 in Supplementary Material). This is likely a consequence of the fluctuation in spectral ion counts between different samples and could be a due to poor ionization of the tryptic fragments or dissociation from the membrane. However, since ER-AP is predicted to be ER-associated and it has been shown that the ER is in contact with chloroplasts, the large variability in ER-AP protein abundance may be a result of different degrees of chloroplast-ER interactions (Andersson et al., 2007).

The direct relationship between gene expression and protein abundance, however, is not true for all proteins: the two proteins, which were assigned to the BS, based on spectral ion counts, show equal gene expression levels in both M and BS cells. In the case of Hyp3, this may be due to a generally small amount of the protein within our samples. On the other hand, while Mep3 protein is present in equal quantities in BS and M cell, gene expression appears to be increased in BS cells. Given that several of these proteins had been detected in chloroplasts by other groups, contamination seems unlikely. On the other hand, it has been shown that protein abundance and mRNA levels do not necessarily correlate, especially in plastids (Li et al., 2010). These authors calculated BS versus M localization based on RNA-Seq data and compared those to a proteomics data set (Friso et al., 2010). They assigned ER-AP, UP-d, and PEPC to the mesophyll, while Mep1, Mep3, and NADP-ME were allocated to the BS cells. Yet, they found correlation between their data and protein abundances in some but not all cases (for example BS/M ratio for Mep1: 0.8 based on proteomics, 2.0 based on RNA-Seq). Possible explanations could be that either mRNA or proteins are more stable in the BS or that the protein is not imported into the mesophyll envelope. This would suggest further mechanisms controlling the turnover and incorporation of chloroplast envelope proteins.

CONFIRMATION OF CHLOROPLAST LOCALIZATION OF SELECT CHLOROPLAST ENVELOPE PROTEINS

To confirm the chloroplast localization of the above proteins, we cloned the respective genes with a carboxy-terminal GFP or YFP tag using the Gateway cloning system and transiently expressed them in *Nicotiana tabacum* (**Figure 4**). ER-AP, Mep 3, UP-a, Hyp g, and Hyp d show an even co-localization with the chloroplast, suggesting they are present in the plastid. PIC and 5TM show a spotted pattern that is clearly associated with the chloroplast but appears to be on the surface of the plastid. The pattern is similar to the one observed for multiple inner envelope proteins (Breuers et al., 2012).

DISTRIBUTION OF GENE EXPRESSION THROUGHOUT THE PLANT AND DURING TRANSITION TO LIGHT

To better understand if and how 5TM, ER-AP, UP-d, and PIC could be associated with chloroplast development and/or function in relation to C4 photosynthesis, we studied their gene expression throughout the plant as well as at different times during leaf

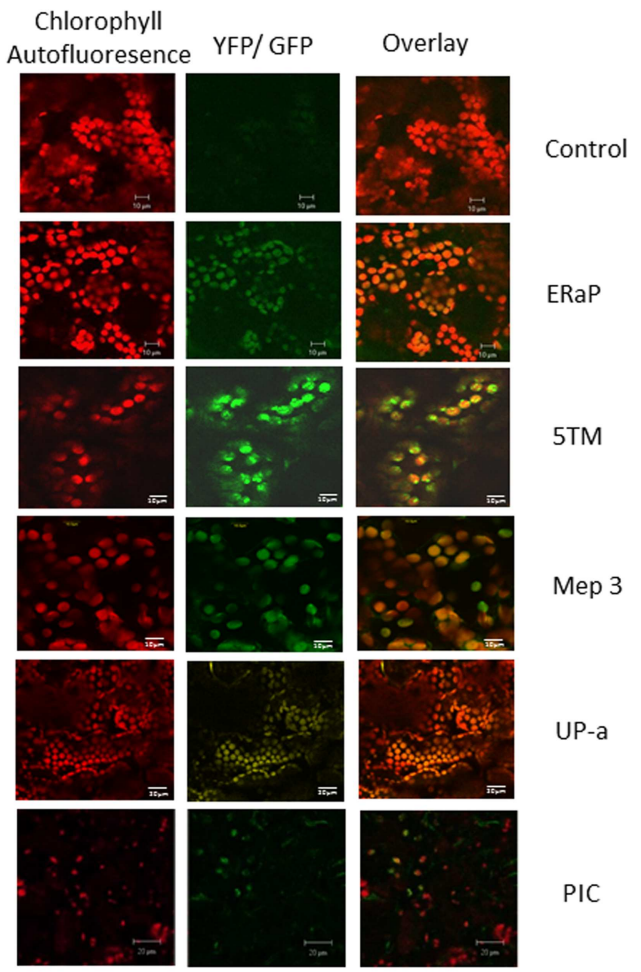

FIGURE 4 | Localization of chloroplast envelope proteins with C-terminal GFP or YFP tags that were transiently expressed in *Nicotiana tabacum*. The first column shows chlorophyll autofluorescence, the second column GFP or YFP fluorescence, column three shows the overlay. Protein names (as used in **Table 1**) are indicated on the right of each row.

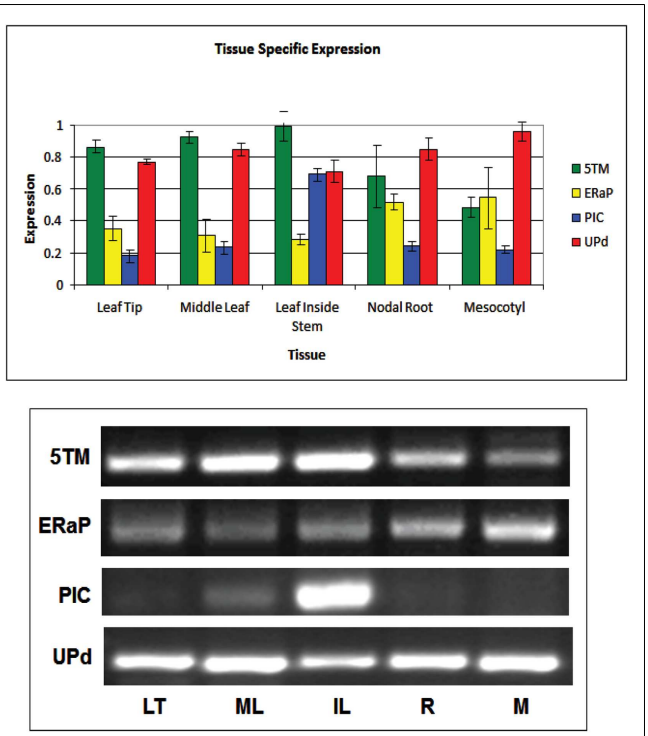

FIGURE 5 | Quantification of semiquantitative RT-PCR showing tissue-specific expression of genes encoding the 5TM protein (green bars), ER-AP (yellow bars), PIC-like protein (blue bars), and UP-d (red bars). Representative gel pictures are shown in the lower half. Band intensities of three to five biological replicates were quantified and displayed in the bar graph.

development and normalized them based on 18S expression levels (**Figure 5**). Leaf samples were taken at the tip (fully developed and expanded; source tissue; part 1 and 2 according to Pick et al., 2011), center (fully developed; expanding; source tissue; part 5 according to Pick et al., 2011), and at the base inside the sheath (etiolated; expanding; sink tissue). To investigate a possible role in chloroplast development, expression was also monitored during the transition of 5-day-old dark-grown seedling into light (**Figure 6**). Primary leaves in these seedlings started to turn green within 3–5 h, a process that was completed by 24 h.

The gene for 5TM shows high expression in all parts of the leaf but is also expressed in the root and the mesocotyl in 6-week-old plants. Similarly, if 6-day-old etiolated corn seedlings are moved to light, 5TM gene expression in the leaves is constitutively high over a 30-h time period after transition to light (**Figures 5** and **6C**). This suggests its function likely is not related to photosynthesis. Similarly, there is no significant change of Up-d expression during transition to light (**Figure 6D**).

PIC expression is present mostly in parts of the leaf that were located within the sheath and not exposed to light and to a much smaller extent in the green part of the leaf (**Figure 5**). During transition to light, it shows a transient 60% increase for the first 8 h before being gradually reduced to 50% of the expression at the start of the exposure (**Figure 6A**). This could indicate a role either in chloroplast development or in processes that are present in the dark and cease after transition to light.

ER-AP gene expression appears to be slightly higher in roots and mesocotyl than in leaves (**Figure 5**). When moved to light, however, ER-AP expression more than doubled within 4–6 h, followed by a return to dark-grown levels within the next 6 h (**Figure 6B**). This increase was statistically significant ($p < 0.05$ for the 4, 8, and 10-h time points and $p < 0.1$ for the 6-h time point). This suggests that while the gene product may be necessary for general cellular functions, it may also be relevant for the light-dependent transition from proplastids to chloroplasts.

CONCLUSION

A large number of chloroplast proteins and putative metabolite transporters have already been identified through proteomics experiments. In addition, genome databases have increased the number of candidates. We have shown here that despite this large

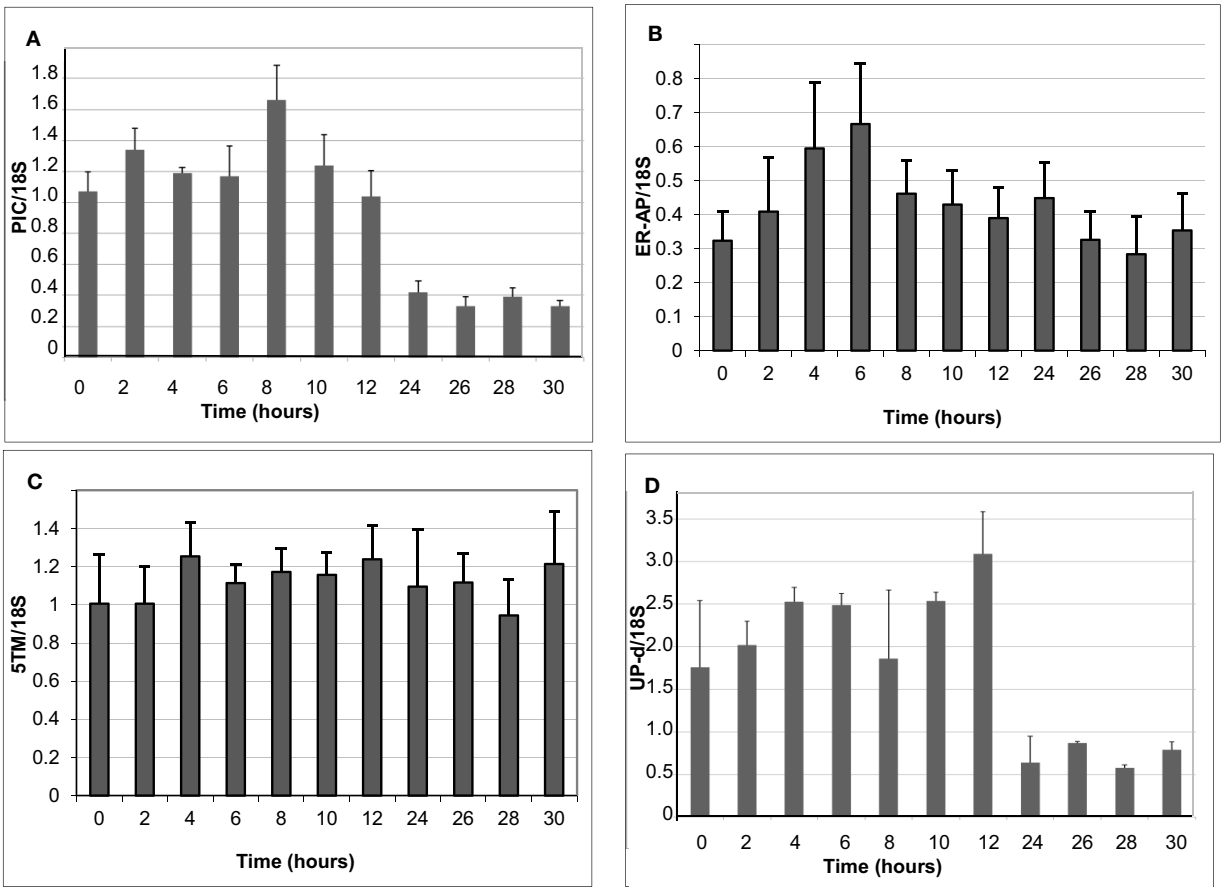

FIGURE 6 | Change in the expression of genes encoding the PIC-like protein (A), ER-AP (B), 5TM protein (C), and UP-d (D). Values were obtained from five to six biological replicates.

candidate pool, further fractionation can still lead to the discovery of novel proteins. To make these protein lists meaningful, it is now necessary to characterize bioinformatics predictions. We have confirmed the chloroplast association of seven of our identified chloroplast envelope proteins. Based on gene expression studies throughout the plant and during transition to light, we conclude that the 5TM and the UP-d protein may not relevant for chloroplast development or C4 metabolite transport, but that ER-AP and PIC constitute good candidates for further study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Proteomics/10.3389/fpls.2013.00065/abstract

Table S1 | Primers used for RT-PCR experiments.

Table S2 | Proteins identified in bundle sheath and mesophyll envelopes.

Table S3 | Raw data and calculation of BS/M ratio based on spectral ion count.

Table S4 | Proteins with differential abundance that are depicted in Figure 2.

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Fusarium graminearum and its interactions with cereal heads: studies in the proteomics era

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The ascomycete fungal pathogen *Fusarium graminearum* (teleomorph stage: *Gibberella zeae*) is the causal agent of *Fusarium* head blight in wheat and barley. This disease leads to significant losses of crop yield, and especially quality through the contamination by diverse fungal mycotoxins, which constitute a significant threat to the health of humans and animals. In recent years, high-throughput proteomics, aiming at identifying a broad spectrum of proteins with a potential role in the pathogenicity and host resistance, has become a very useful tool in plant-fungus interaction research. In this review, we describe the progress in proteomics applications toward a better understanding of *F. graminearum* pathogenesis, virulence, and host defense mechanisms. The contribution of proteomics to the development of crop protection strategies against this pathogen is also discussed briefly.

Keywords: *Fusarium graminearum*, *Fusarium* head blight, pathogenicity, plant defense response, proteomics

INTRODUCTION

The pathogen *Fusarium graminearum* causes devastating head blight of small grain cereals including wheat and barley. *Fusarium* head blight (FHB), as a global problem, has great economic impact on the cereal industry due to the reduced grain yield and quality as well as to the contamination by diverse mycotoxins, including deoxynivalenol (DON) and zearalenone, which are harmful for humans and animals.

The disease (**Figure 1A**) is initiated by deposition of spores on or inside flowering spikelets (Bushnell et al., 2003). Fungal hyphae develop on the exterior surfaces of florets and glumes, rather than by direct penetration through the epidermis, prior to the colonization of anthers, stigmas, and lodicules (Bushnell et al., 2003). The fungus spreads in wheat from spikelet to spikelet through the vascular tissue in the rachis and rachilla (Trail, 2009) and this is associated with the production of DON, a virulence factor (effector molecule) causing tissue necrosis (Jansen et al., 2005). In barley, spread of the disease is limited and virulence does not appear to be due to the presence of the toxin (Maier et al., 2006). The fungus apparently exhibits a brief biotrophic phase before switching to the necrotrophic phase, when vigor of colonization increases by the fungus and eventually the plant cells die (Trail, 2009).

As a result of its devastating effects, *F. graminearum* has been under intense investigation for many years to understand the genetic basis of the life cycle, pathogenicity, evolution, and population biology. Availability of the full genome sequence (Ma et al., 2010) considerably revitalizes research of gene function in *F. graminearum*. In addition to classical biochemical, genetic, molecular biological, and plant pathology approaches, several "omics" techniques are employed in the studies of *F. graminearum* and its interactions with hosts. Transcriptome and metabolome analysis have been conducted in *F. graminearum* during the invasion of hosts, sexual development, and conidial germination, in response to azole fungicide and/or in *F. graminearum* mutants as well as in barley and wheat during infection to understand defense responses (reviewed by Kazan et al., 2012). *In silico* prediction of the secretome of *F. graminearum* has also been performed to identify potential pathogenicity factors and effectors (Brown et al., 2012). Proteomics, as the core technology in functional genomics, allows interpretation of gene function, determination of protein abundance, interactions, modifications, locations, and implications in development and environmental responses (Wright et al., 2012). In the present review, we focus on the recent progress made by using proteomics techniques to enhance the understanding of cellular and molecular mechanisms of *F. graminearum* pathogenicity and virulence as well as the host defense responses.

PROTEOMICS TECHNIQUES IN PHYTOPATHOGENIC FUNGI

Proteome analysis of phytopathogenic fungi and their interactions with hosts has increased dramatically over the last years, because of the technical development of "omics" and bioinformatic tools, and the growing number of fungal genomes being sequenced. Investigations in this area mainly are (i) identification of mycelial, conidial, and secreted proteins across a range of fungal species by establishing reference proteome maps of these fungal structures. Proteome profiles are conducted and/or compared between species, races, mutants, growth, development stages, and growth conditions (Gonzalez-Fernandez et al., 2010), in particular during spore germination, hyphal penetration, appressorium formation, toxin production, and secretion (vanKan, 2006), and (ii) plant-fungus interactions to study infection cycles, to identify pathogenicity factors and to study plant defense responses.

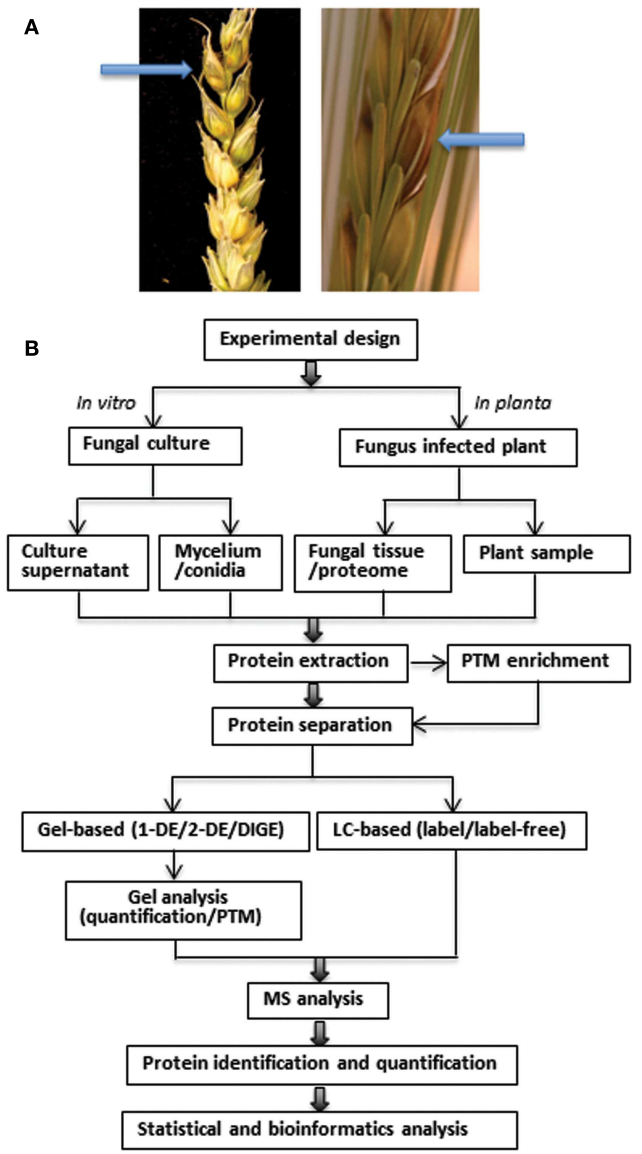

FIGURE 1 | (A) Symptoms (indicated by arrows) of *Fusarium* head blight in the spikelets of wheat (left) and barley (right). Bleaching and dark necrotic lesions can be observed in the infected wheat spikelets. Infected barley spikelets show a browning or water-soaked appearance. The spikelets were point-inoculated with *F. graminearum* at anthesis and photographed at 6 dai by Jens Due Jensen and David B. Collinge, University of Copenhagen. **(B)** Schematic overview of proteomics workflow in phytopathogenic fungi. The major steps include experiment design, sampling, protein extraction, PTM enrichment, protein separation, MS analysis, protein identification, and quantification, followed by bioinformatics analysis of the data.

Analysis of proteins of some fungal species *in planta* is limited due to the fact that it is difficult to isolate fungal tissues from the infected hosts and that the fungal biomass constitutes a small portion of the total biological material resulting in the dominance of plant proteins. Besides fungi with agricultural interest, such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *F. graminearum* (reviewed by Gonzalez-Fernandez and Jorrin-Novo, 2012), important studies employing a diversity of proteomics techniques have been performed on major crops, including rice, maize, wheat, and barley interacting with fungal pathogens, in addition to *Arabidopsis thaliana* (reviewed by Gonzalez-Fernandez et al., 2010).

The workflow of proteomic analysis in phytopathogenic fungi is shown in **Figure 1B**. Experimental design and sampling reflect the aim of the study, i.e., whether it has focus on the fungus or the plant under the chosen conditions. The protein extraction protocol is a very critical step and determines which proteins are available for analysis. This step is particularly challenging for both plants and fungi, because of their robust cell walls in addition to proteases and different non-protein components which can interfere both with the population and quality of the proteins and their subsequent separation (Hurkman and Tanaka, 2007). Post-translational modifications (PTMs) can also be analyzed using proteomics, but require selective enrichment and purification strategies due to their reversible and labile nature and low stoichiometric abundances. Some PTMs, such as phosphorylation, glycosylation, acetylation, phenylation, *S*-nitrosylation, and ubiquitylation, are involved in signal transduction during plant-microbe interactions and have been analyzed by proteomics (Jayaraman et al., 2012).

Protein separation in the majority of earlier proteomics studies was based on two-dimensional gel electrophoresis (2-DE) coupled with conventional staining methods. Difference gel electrophoresis (DIGE), where samples are labeled differentially with fluorophores, allows distinction between proteins obtained in different samples that can be resolved on the same gel. This can address the issues of both sensitivity and gel variability in 2-DE (Wright et al., 2012). However, DIGE suffers from the same problems as traditional 2-DE, especially in relation to the resolution of hydrophobic proteins and proteins exhibiting extreme pIs and molecular weights. Currently, gel-free techniques for separating peptides become standard for large-scale shotgun proteomics, which can overcome some of the limitations of the gel-based approach. The methods are based on the pre-fractionation of peptide mixtures by mono-dimensional LC or multidimensional protein identification technology (MudPIT) such as strong cation exchange (SCX) combined with reversed phase chromatography (Gilmore and Washburn, 2010).

Mass spectrometry (MS), consisting of an ion source, a mass analyzer, and a detector, is the most common technique for unbiased protein identification (Aebersold and Mann, 2003). The various techniques for ionizing samples include matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The mass analyzers include time-of-flight (TOF), ion trap, quadruple, orbitrap, and fourier transform ion cyclotron resonance. In MS/MS, specific precursor ions produced in the initial mass analyzer are chosen and fragmented, resulting in sequence-informative fragment ion spectra. Fragmentation methods can be collision-based (e.g., CAD and HCD) or electron-based (e.g., ECD and ETD) dissociation (Coon, 2009). Observed ion spectra are compared against databases containing known protein sequences by search algorithms (e.g., SEQUEST, Mascot, and OMSSA) for protein identification.

Comparative proteomics can be based on the traditional pre-staining of 2-DE gels such as Coomassie Blue staining, silver staining, and fluorescence staining and the modern label-free or labeling approaches at the MS stage, followed by the statistical, and bioinformatics analysis to determine the significance of data. Isotope-assisted quantification methods include *in vitro* chemical (e.g., ICAT, iTRAQ, TMT, and ^{18}O) and *in vivo* metabolic (e.g., SILAC and ^{15}N-labeling) labeling of biological samples. In chemical labeling, distinct protein samples are labeled with heavy and light isotopes or isobaric tags, pooled, and compared by MS. Stable isotope labeling of amino acids in cell culture (SILAC) or plants that are grown on media supplemented with heavy isotope-containing amino acids, allows for labeling of proteins as they are synthesized (Ong et al., 2002). The relative ratio of protein from different samples is determined by the ratios of signal intensities of the labeled peptides that are common to the samples in MS analysis. Label-free quantification compares samples based on the measurement of changes in peptide peak areas or peak heights in chromatography and peptide peak intensity in MS or the spectral counting of identified proteins after MS/MS analysis (Neilson et al., 2011).

FUSARIUM GRAMINEARUM PROTEOME ANALYSES

Proteomics studies conducted on *F. graminearum* have focused mainly on the secretome and impact of DON (**Table 1**). This is due to the important roles of secreted proteins and DON in pathogenicity. The first *in vitro* gel-based secretome study in *F. graminearum* was performed in a culture with a medium containing either glucose or hop cell walls. Here, 23 and 84 unique proteins were identified, respectively, mainly involved in cell wall polysaccharide degradation (Phalip et al., 2005). Using LC-MS/MS, 229 fungal proteins, mostly glycoside hydrolases and proteases, were identified in the secretome of *F. graminearum* during growth on 13 synthetic media (Paper et al., 2007). To closely mimic the nutritional situation of the fungus *in planta*, Yang et al. (2012) employed a gel-based proteomics approach to access the secretome in the growth cultures with barley or wheat flour as the sole nutrient source, resulting in the identification of 69 unique fungal proteins including enzymes involved in the degradation of cell walls, starch, and proteins. Secreted proteins differing in accumulation between wheat and barley flour media were mainly involved in fungal cell wall remodeling and the degradation of plant cell walls, starch, and proteins. To analyze the effect of DON production in host infection process, *F. graminearum* was grown on a medium promoting trichothecene biosynthesis (Taylor et al., 2008). Here, comparative proteomics showed 130 differentially expressed fungal proteins, of which proteins potentially involved in virulence were up-regulated, whereas down-regulated proteins were primary metabolic enzymes, chaperones, and proteins involved in translation.

Two phosphoproteome studies of *F. graminearum* under nitrogen limiting conditions and under conditions of unlimited nutrients have been published recently (Rampitsch et al., 2010, 2012). It was suggested that phosphorylation events are involved in the signaling pathways, leading to the activation of the trichothecene pathway, which is also activated in *F. graminearum* under nutrient stress (Rampitsch et al., 2010). A total of 348 phosphorylation sites localized to 301 peptides from 241 proteins including 10 protein kinases and seven transcription factors were identified during nitrogen starvation. When *F. graminearum* was grown *in vitro* without nutritional limitation, 2902 putative phosphopeptides with homologous matches to 1496 different proteins were identified (Rampitsch et al., 2012). Here, the majority of phosphoproteins were nuclear proteins with ATP-binding function and the phosphorylation sites were conserved in three phosphopeptides from transcription elongation factor 1β, acidic ribosomal proteins, and glycogen synthase.

Although it is very challenging to identify large numbers of *F. graminearum* proteins *in planta*, Paper et al. (2007) extracted *F. graminearum* secreted proteins from infected wheat heads by vacuum filtration, resulting in the identification of 120 fungal proteins including several cell wall degrading enzymes, of which 56% contained putative secretion signals. Additionally, proteomics analyses of *F. graminearum*-infected barley spikelets at maturity (Yang et al., 2010a) and 2 days after inoculation (dai; Yang et al., 2010b) as well as wheat spikelets from 1 to 3 dai (Zhou et al., 2006), revealed nine, one, and eight fungal proteins, respectively. The identification of fungal stress-related and antioxidant proteins *in planta* strongly suggests that the pathogen is exposed to stresses such as oxidation and starvation and that it attempts to overcome plant defense.

PROTEOMICS STUDIES OF HOST DEFENSE TO *FUSARIUM GRAMINEARUM*

Extensive proteomics studies have been conducted in *F. graminearum*-infected wheat, barley, and their wild relatives (**Table 1**). With the exception of one recent study (Gunnaiah et al., 2012) using shotgun proteomics, other studies have employed gel-based techniques to investigate the differentially expressed proteins of hosts with different levels of disease susceptibility at different time points after inoculation at anthesis or during germination (**Table 1**). Due to the use of different cultivars, inoculation methods, infection stages, growth conditions, and proteomic techniques, little overlap is apparent between the regulated proteins identified in these studies. In resistant and/or susceptible wheat in response to *F. graminearum* up to 5 dai, many proteins related to carbon metabolism and photosynthesis were down-regulated, whereas the up-regulated proteins could be involved in antioxidant, jasmonic acid, and ethylene signaling pathways, phenylpropanoid biosynthesis, antimicrobial compound synthesis, detoxification, cell wall fortification, defense-related responses, amino acid synthesis, and nitrogen metabolism. Wheat susceptibility likely reflected the delayed activation of the salicylic acid defense pathway (Ding et al., 2011). Moreover, distinct abundance patterns of different xylanase inhibitor forms and pathogenesis-related (PR) proteins were shown in the wheat ear in response to the *F. graminearum* Δ*Tri5* mutant at 5, 15, and 25 dai (Dornez et al., 2010).

When the proteomes of mature grains of susceptible barley infected by *F. graminearum* under two different levels of nitrogen fertilizers were analyzed, massive, fungus-induced degradation of the grain proteome was observed and increased *Fusarium* infection occurred with low N amount (Yang et al., 2010a). In contrast, Zantinge et al. (2010) observed no degradation of seed proteomes

Table 1 | Original proteomics papers published on *F. graminearum* and its interactions with wheat and barley.

Growth conditions	Sampling times	Sample materials	Proteomics techniques	Remarks	Reference
In the growth media containing either glucose or hop cell walls	6 d, 9 d	Culture supernatants	1-DE, 2-DE, LC-MS/MS	Analysis of the fungal *in vitro* secretomes	Phalip et al. (2005)
In the synthetic media containing polysaccharide supplements	7 d	Culture supernatants	1-DE, LC-MS/MS	High-throughput analysis of the fungal *in vitro* secretomes	Paper et al. (2007)
In the wheat grains	Maturity	Fungal secretome	1-DE, LC-MS/MS	Analysis of the fungal *in planta* secretomes	Paper et al. (2007)
In the growth medium promoting trichothecene biosynthesis after 2-day growth in the rich medium	0, 4 d, 9 d, 12 d	Fungal tissues	iTRAQ, LC-MS/MS 2-DE, MS/MS	*In vitro* time course study of the changes in fungal intercellular proteomes due to the induction of trichothecene production	Taylor et al. (2008)
In the growth media containing only barley or wheat flour	7 d	Culture supernatants	2-DE, MALDI-MS/MS	Study of the fungal *in vitro* secretomes under growth conditions which mimic *in planta* nutritional situation	Yang et al. (2012)
In the growth medium with limited nitrogen after 2-day growth in the rich medium	0, 6 h, 12 h	Fungal tissues	2-DE, MALDI-MS, 1-DE, IMAC, TiO_2, LC-MS, SAX, IMAC, LC-MS/MS	Analysis of the fungal phosphoproteomes under the *in vitro* growth condition that activates trichothecene pathway	Rampitsch et al. (2010)
In the growth medium with unlimited nutrients	1 d	Fungal tissues	SCX, IMAC, LC-MS/MS	Analysis of the fungal *in vitro* phosphoproteomes	Rampitsch et al. (2012)
Virus-free and -infected strains grown in the complete medium	5 d	Fungal tissues	2-DE, LC-MS/MS	Study of the fungal proteomes in response to viral infection	Kwon et al. (2009)
In the resistant wheat spikes	6 h, 12 h, 24 h	Wheat spikes	2-DE, MALDI MS	Study of the differential expressed wheat proteins in response to fungal infection	Wang et al. (2005)
In the susceptible and resistant wheat spikes	5 d	Wheat spikelets	2-DE, LC-MS/MS	Study of the differential expressed wheat proteins in response to fungal infection	Zhou et al. (2005)
In the susceptible wheat spikes	1 d, 2 d, 3 d	Wheat spikelets	2-DE, LC-MS/MS	Identification of wheat proteins regulated by the fungus and fungal expressed proteins *in planta*	Zhou et al. (2006)
In the susceptible wheat ears	5 d, 15 d, 25 d	Wheat ears	DIGE, MALDI MS/MS	Investigation of the changes in xylanase inhibitors (iso)forms of wheat due to fungal *Tir5* mutant infection	Dornez et al. (2010)
In the susceptible and resistant wheat spikes	12 h	Wheat spikes	2-DE, MALDI MS	Study of the differential expressed wheat proteins and genes in response to fungal infection	Ding et al. (2011)
In the moderate resistant wheat spikes	48 h	Wheat spikes	2-DE, MALDI MS	Study of the differential expressed wheat proteins in response to fungal infection	Shin et al. (2011)

(Continued)

Table 1 | Continued

Growth conditions	Sampling times	Sample materials	Proteomics techniques	Remarks	Reference
In wheat carrying either resistant or susceptible alleles at the *Fhb 1* locus	72 h	Wheat spikelets	LC-MS/MS, spectral counting	Identification of mechanisms of resistance governed by the FHB resistance locus *Fhb 1*	Gunnaiah et al. (2012)
In emmer heads and co-colonization with *Fusarium culmorum*	Maturity	Emmer grains	2-DE, LC-MS/MS	Study of the differential expressed emmer seed proteins in response to fungal infection	Eggert et al. (2011)
In the spikes of six barley genotypes of varying resistance	3 d	Barley spikelets	2-DE, LC-MS/MS	Study of the differential expressed barley proteins in response to fungal infection	Geddes et al. (2008)
In the susceptible barley spikes grown under different N fertilizers	Maturity	Barley seeds	2-DE, MALDI MS/MS	Investigation of effect of nitrogen fertilizer mounts on the severity of FHB and identification of fungal proteins *in planta*	Yang et al. (2010a)
In the susceptible barley spikes	2 d	Barley spikelets	2-DE, MALDI MS/MS	Definition of infection levels correlated to fungal induced plant proteome degradation and identification of the differential expressed barley proteins in response to fungal infection	Yang et al. (2010b)
In the susceptible barley seeds	3 d	Germinating barley seeds	2-DE, MALDI MS/MS	Study of the differential expressed barley seed proteins in response to fungal infection during germination	Yang et al. (2011)
In the spikes of eleven barley genotypes of varying resistance	Maturity	Barley seeds	2-DE, LC-MS/MS	Study of the differential expressed barley seed proteins in response to fungal infection	Zantinge et al. (2010)
In the naked barley heads and co-colonization with *Fusarium culmorum*	Maturity	Naked barley grains	2-DE, MALDI MS, LC-MS/MS	Identification of the differentially expressed seed proteins in response to fungal infection and to growing location of the plant	Eggert and Pawelzik (2011)

of barley cultivars with different susceptibility to *F. graminearum*. These findings led to the analysis of the compatible interaction between barley and *F. graminearum* during the early infection stage to clearly define the infection levels correlated to the degree of fungal induced proteome degradation. Analysis of infected susceptible barley spikelets at 2 dai (Yang et al., 2010b) and 3 dai (Geddes et al., 2008) revealed up-regulation of proteins associated with oxidative stress response, PR-proteins and increased energy metabolism, although slight proteome degradation was observed at 2 dai (Yang et al., 2010b). However, changes in proteins associated with an oxidative response could not be observed in resistant barley (Geddes et al., 2008), suggesting enhanced oxidative stress in the compatible interaction. Moreover, investigation of infection of emmer wheat and naked barley mature grains by both *F. graminearum* and *F. culmorum* showed DON accumulation and several changed proteins involved in transcription regulation, defense responses, nutrient reservoirs, and starch biosynthesis in contrast to proteome degradation (Eggert and Pawelzik, 2011; Eggert et al., 2011). These results indicate that wild relatives can stimulate defense strategies in response to *Fusarium* infection after a long infection period up to the maturity stage.

CONTRIBUTION OF PROTEOMICS TO CROP PROTECTION AGAINST FHB

In order to reduce epidemics of FHB, several approaches for control can be employed, including the use of cultural control techniques (e.g., crop rotations, irrigation, weed control, nitrogen input, and tilling), the use of fungicides, chemicals and biological control, transgenic plants, and resistance breeding (Parry et al., 1995). The major contribution of proteomics to crop protection is the identification of both fungal effectors possibly facilitating infection or triggering plant defense and host proteins or biomarkers possibly conferring enhanced resistance, which require subsequent functional analysis of corresponding genes to establish new strategies for disease control. So far, several *F. graminearum* genes relative to mycotoxin production, signal transduction, metabolism, and growth have been analyzed in detail to examine their roles in the virulence and pathogenicity (reviewed by Kazan et al., 2012), but the targets discovered on the basis of the outcomes of proteomics, which may be essential for fungal infection, have not been well investigated.

With respect to host resistance, proteomics has identified many host proteins in response to *F. graminearum*, the majority of which are often involved in primary metabolism, defense, and stress-related responses. However, the most frequently identified host proteins have not been fully investigated, except the PR-proteins (e.g., chitinase, β-1,3-glucanase and thaumatin-like protein), in terms of downstream characterization of their functional roles in enhanced resistance. The reasons can be that some proteins of interest are actually not found by proteomics due to low abundance or condition-dependent expression and that high-throughput stable transformation of wheat and barley for functional analysis of host genes is still not available. Furthermore, there has been a lack of wheat and barley genome sequences, although barley genome has recently been published (The International Barley Genome Sequencing Consortium, 2012), which should assist the process of identifying elements relating to resistance. Transgenic wheat expressing a α-1-purothionin, a thaumatin-like protein 1, a β-1,3-glucanase (MacKintosh et al., 2007), a class II chitinase (Shin et al., 2008), an antifungal plant defensin (Li et al., 2011), a pectin methyl-esterase inhibitor (Volpi et al., 2011), a polygalacturonase-inhibiting protein (Ferrari et al., 2012), a lactoferrin (Han et al., 2012), a *Arabidopsis thaliana* NPR1 (Makandar et al., 2006), or a truncated form of the yeast ribosomal protein L3 (Di et al., 2010) enhanced resistance to FHB under greenhouse conditions. However, transgenic lines have rarely been tested for FHB severity under field conditions. Among tested lines, a β-1,3-glucanase and a L3 transgenic wheat line conferred enhanced resistance in addition to reduced DON level in the L3 line. Transgenic barley expressing a FsTri101, a PDR5, a chitinase, or a thaumatin-like protein showed that neither of these genes was effective in the field at reducing FHB or DON levels, whereas two transgenic lines expressing another thaumatin-like protein or a trichothecene transporter, have shown reduced DON accumulation during 5 years of field trials (Dahleen et al., 2011). FHB resistance breeding is another approach, where mapping of QTLs controlling resistance to *F. graminearum* is a major task. Resistance-associated host metabolite-based breeding selection has also been suggested, when reliable associations between these metabolites as biomarkers and host resistance can be established (Bollina et al., 2011). The same principle can be applied to genes and proteins. Large-scaled "omics" studies of host-*F. graminearum* interactions offer such opportunity to identify potential biomarkers.

PERSPECTIVES

Proteomics has become an indispensable tool for understanding molecular and cellular mechanisms in plant-microbe interactions. With the aid of the remarkable development of proteomics techniques, host genome sequencing and bioinformatics tools, the capability of proteomics to identify the novel elements involved in *F. graminearum* pathogenicity and virulence and host resistance will continue to improve. However, the full characterization of a proteome is extremely challenging due to proteome dynamics and complexity. The high cost and complexity of experimental procedures also limit the utilization of proteomics. Furthermore, functional analysis of identified proteins or genes is required to elucidate their roles in pathogenicity and plant resistance. Although significant progress has been made in understanding FHB, environmental changes, and evolution of virulence and toxin biosynthesis in *F. graminearum* are highly challenging for disease control. Therefore, it will be essential to integrate all the information generated from the "omics" studies together with plant pathology and genetic engineering to fully understand host *F. graminearum* interactions for development of sustainable cereal protection strategies.

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Proteomic insights into intra- and intercellular plant–bacteria symbiotic association during root nodule formation

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Over the last several decades, there have been a large number of studies done on the all aspects of legumes and bacteria which participate in nitrogen-fixing symbiosis. The analysis of legume–bacteria interaction is not just a matter of numerical complexity in terms of variants of gene products that can arise from a single gene. Bacteria regulate their quorum-sensing genes to enhance their ability to induce conjugation of plasmids and symbiotic islands, and various protein secretion mechanisms; that can stimulate a collection of chain reactions including species-specific combinations of plant-secretion isoflavonoids, complicated calcium signaling pathways and autoregulation of nodulation mechanisms. Quorum-sensing systems are introduced by the intra- and intercellular organization of gene products lead to protein–protein interactions or targeting of proteins to specific cellular structures. In this study, an attempt has been made to review significant contributions related to nodule formation and development and their impacts on cell proteome for better understanding of plant–bacterium interaction mechanism at protein level. This review would not only provide new insights into the plant–bacteria symbiosis response mechanisms but would also highlights the importance of studying changes in protein abundance inside and outside of cells in response to symbiosis. Furthermore, the application to agriculture program of plant–bacteria interaction will be discussed.

Keywords: proteomics, legumes, bacteria, symbiosis, nodule development

INTRODUCTION

Mutualistic symbiosis includes a wide range of interactions among a diverse set of organisms. The symbiosis between legumes and rhizobia is a classic mutualistic relationship and nitrogen fixation process is one of the most important biological processes on the earth. The symbiosis culminates in the creation of a highly specialized plant organ, the root nodule, with plant cells invaded by bacteria. More than a century ago, Hellriegel and Wilfarth (1888) identified rhizobia as a source of nitrogen fixation. Rhizobia, Gram-negative soil bacteria, induce the formation of nodules in many, but not all, leguminous plants (Gualtieri and Bisseling, 2000). The nitrogen fixing nodule is a model for plant developmental processes and plant–microbe interactions. The nodule forms an anaerobic niche for nitrogen fixation, protecting the bacterial nitrogenase from inactivation by O_2. In exchange for carbohydrates provided by the host legume, fixed nitrogen would be supplied by bacteria to the legume.

Establishment of symbiosis between host plants and symbiotic bacteria is a multistage process covering signal perception, transduction, and responses (Broughton et al., 2000). These processes depend on the precise spatial and temporal regulation of nod- and other symbiotic genes (Schlaman et al., 1998). Synchronized expression of symbiotic loci in legumes and their bacterial partner involves the exchange of a series of molecular signals allowing rhizobia to invade the plant roots. Rhizobia produce various molecular signals that influence the host plants at various steps along the symbiotic pathway (Perret et al., 2000). Symbiosis is initiated when accumulated flavonoids in the rhizosphere of the host plant prompt the cascade of rhizobial signal transduction by interacting with transcriptional activators of nodulation genes. This flavonoid-modulated signal transduction cascade regulates expression of genes that act during nodule development. Most nodulation genes including nol, noe, and nod, are involved in the synthesis of host-specific lipochitooligosaccharides (Nod factors), which are essential for the initial infection of root hairs (Perret et al., 2000). Nod factors provoke root curling, creation of nodule primordia, early nodulin (*ENOD*) genes expression, and finally allow the bacteria to enter the root hairs (Broughton et al., 2000; Geurts and Bisseling, 2002). Thus, flavonoids and Nod factors correspond to the primary sets of signal transduction by the symbiotic partners.

Further, already differentiated cortical cells have to be reactivated and enter the cell cycle from their arrested state, so that a

Abbreviations: GEP, the general export pathway; IT, infection thread; Lbs, leghemoglobins; LCOs, lipochitooligosaccharides; Nop, nodulation outer protein; NSP, nodulation signaling pathway; pbm, peribacteroid membrane; RAP, rhizobium-adhering proteins; RNAi, RNA interference; SNF, symbiotic nitrogen fixation; TAT, twin-arginine translocation pathway; TF, transcription factors.

nodule primordium is formed. Release of the bacteria into primordium cells results in its differentiation into a nodule (Gage, 2004; Oldroyd and Downie, 2004). The infection process as well as the induction of cortical cell divisions is caused by Nod factors that are secreted by rhizobia when they colonize the roots of their host. In legumes, the infection process starts in epidermal root hair cells (Brewin, 2004). Nod factor-secreting rhizobia induce deformations in most of the root hairs in a region of the root that is susceptible to the interaction (Heidstra et al., 1994). In this way, the bacterium becomes entrapped within a three-dimensional cavity of curl and a small colony of rhizobia is formed. Subsequently, these rhizobia induce local weakening of the cell wall and, by invagination of the plasma membrane of the root hair, a tube-like structure is formed. This is the so-called infection thread (IT) that allows the rhizobia to penetrate the root-hair cell. Each root cortical cell that traversed to make an IT and ultimately the rhizobia reach the nodule primordium (Brewin, 2004). Cortical roots taken up in the nodule primordium cells in an endocytosis-like manner forming organelle-like structures (symbiosomes) that contain one or more bacteria, which upon differentiation start to fix nitrogen (Brewin, 2004).

Proteomics is a high-throughput technology that has been used to investigate a wide range of biological aspects including phylogenetic and molecular divergence studies (Bushehri et al., 2008, 2011), plant responses to different stresses (Larrainzar et al., 2007; Danchenko et al., 2009; Klubicova et al., 2012; Komatsu et al., 2012; Mohammadi et al., 2012; Salavati et al., 2012b) detailed studies on the structural components, and biochemical pathways involved in symbiotic nitrogen fixation (SNF; Broghammer et al., 2012; Navascués et al., 2012; Rose et al., 2012). Approaches such as transcriptome, proteome, and metabolome analysis in both symbionts, promise to reveal much more detail about the metabolic flows in the nitrogen fixing nodule or even to description the novel unknown aspects (Delmotte et al., 2010; Khatoon et al., 2012; Salavati et al., 2012a,c). Several proteomic studies focused on characterization of different aspects and stages of plant–microbe interactions were published. Natera et al. (2000) identified root nodule proteins in *Melilotus alba* during 12 days after inoculation by *Sinorhizobium meliloti*, including *S. meliloti* and bacteroid proteins. Proteins involved in nodule formation and regulated by auxin were identified in *Medicago truncatula* infected by *S. meliloti* (van Noorden et al., 2007). Other previous studies described proteomes of microbial symbionts. Analysis of *hfg* mutant *S. meliloti*, drastically affected in its ability to colonize and initiate symbiosis, showed that ABC type transporter system represents most abundant class of differentially expressed proteins (Barra-Bily et al., 2010). Extracellular proteome of *Rhizobium etli* strain during different growth stages was described by Meneses et al. (2010). Their results suggests that secretome of *R. etli* consists of actively secreted proteins, which mostly are extracellular enzymes (mostly degradation enzymes) and proteins that bind nutrients and extracellular appendages, and proteins that have functions in the cytosol and are not actively secreted but may be released into the culture medium. Function of many identified proteins in extracellular proteome is still unknown.

In addition, the organeller proteins with potential role in the entry of symbiotic bacteria into plant roots or in the other steps of symbiotic processes were also studied (Robertson et al., 1978; Wienkoop and Saalbach, 2003; Hoa et al., 2004; Imaizumi-Anraku et al., 2005). First study focused on phosphoproteome changes during symbiosis was performed by Rose et al. (2012).

Also a few reviews concerning proteomic analysis of plant–microbe interactions in general were published (Rolfe et al., 2003; Bestel-Corre et al., 2004; Mathesius, 2009; Muneer et al., 2012). Our review is more focused to SNF. The integration of genomics and post-genomics events is a strong consensus for functional study of plant–microbe interactions, in general, and SNF, in specific. Model legume genomics and the continued effort on cultivated grain and pasture legumes open unique possibilities for family-based comparative genomics in the Leguminosae. Proteomic studies in combination with transcriptomics studies such as quantitative RT-PCR can advance symbiosis analysis to a new level (Resendis-Antonio et al., 2011; Salavati et al., 2012a). In combination with the on-going genome sequencing and growing EST collection of the model legumes, proteomics has been recently become a powerful investigation of the most detailed physiological events in plant, animal and microorganisms (Colebatch et al., 2004; Thibivilliers et al., 2009). In this paper, we focused on the large-scale identification of proteins and their complexes coupled to genome- and EST-sequence information, which can be used to identify proteins and to monitor changes in protein expression as a function of developmental stages, to review legume nodule initiation and developmental events at translational level.

As technical view, sample preparation is an important step in proteomics researches. This step is principally difficult in studies with plants. Many plant tissues are often rich in proteases contaminants such as polysaccharides, lipids, and phenols (Carpentier et al., 2005). Furthermore, it is necessary to acquire high-quality gels showing reproducible protein patterns (Hurkman and Tanaka, 1986). The extraction method must conserve the quality and quantity of the extracted proteins (Hurkman and Tanaka, 1986; Westermeier and Naven, 2002). Although a single-step process for protein extraction would be highly desirable, no unique sample preparation method can be used to 2-DE analysis (Dunn, 1999). Therefore, many researchers developed and optimized some efficient methods such as a phenol/SDS-based method (Wang et al., 2006; Rodrigues et al., 2012) and non-phenol-based methods (Guerreiro et al., 1997; Natera et al., 2000), to find a simple method that could be applied regularly to proteomics studies of symbiosis interactions.

OVERVIEW OF RHIZOBIUM–LEGUME INTERACTIONS

ROOT ATTACHMENT

Among root-derived compounds, some phenolic-based compounds act as chemotactic attractants and, on the other hand, the secreted and surface proteins are involved in rhizobia attachment to root hairs in the initiation step of the symbiosis (Peters and Verma, 1990; Deakin and Broughton, 2009). Although being in the right place at the right time is critical to the instigation of nodulation, the principal aspects of root attachment, including close contiguity to root hairs, clonal events, and root hair curling, have crucial importance. These steps ensure a supply of nutrients that enable the bacteria to grow on and around the root,

determine whether they will be the ones that can successfully initiate infection in many legumes (Downie, 2010). The fundamentals of communication between the prospective symbiotic partners were established a few years ago (Downie, 1998; Perret et al., 2000; Spaink, 2000). Briefly, the bacteria recognize legumes secretions that passively diffuse across the bacterial membrane (Recourt et al., 1989) via a transcription factor (TF), which typically encoded by nodD. Perret et al. (2000) demonstrated that these Nod factors are primary determinants that decide which legumes will be able to nodulate. Therefore, it has been accepted that the different biovars and species of rhizobia generate a diverse range of Nod factors.

INFECTION INITIATION

Nodule organogenesis begins by an exchange of signals between plant and bacteria, resulting in the curling and colonization of root hairs by rhizobia. Plant-derived membranes then form a tubular structure, called the "infection thread," which guides bacteria to the site of meristematic activity in the root cortex and acts as an effective barrier to confine the bacteria. To analyze the first step of this series of events at the protein level in a time-course study with soybean over the first 48 h, Salavati et al. (2012a) combined 2-D gel electrophoresis coupled with quantitative RT-PCR to analyze isolated proteins at different time points from infected soybean root hairs at both transcriptional and translational levels. Analysis of 56 proteins revealed the differential expression of plant proteins associated with important events, such as metabolism, cell signaling, and disease/defense response. The formation of infected legume nodules capable of fixing nitrogen requires the bacteria to activate two plant programs: one leading to nodule morphogenesis and the other leading to nodule infection. Proteomic techniques were demonstrated that Nod factors can induce nodule morphogenesis, and this appears to occur as a consequence of modifying existing plant hormonal signaling systems, such as the cytokinin pathway (Relic et al., 1994; Hirsch et al., 1997). From a plant perspective, nodule morphogenesis is a critical stage because allowing bacterial entry gives rise to the potential for non-symbiotic bacteria to try to enter and take advantage of the plant (Oldroyd and Downie, 2008). Whereas, bacterial mutants lacking specific nod genes can induce some plant responses such as formation of arrested infections, root hair deformation, plant gene induction, calcium spiking, etc. It seems that producing the correct type of Nod factor by bacteria is critical for successful establishment of ITs (Ardourel et al., 1994; Walker and Downie, 2000; Oldroyd and Downie, 2004).

The growing IT must find this pre-infection thread structure because it allows the IT to join cells, to change from being in the intercellular space, and to promote changes in the direction of its growth. In addition to Nod factor specificity, surface and secretory proteins can also play important roles in infection (Spaink, 2000). The bacteria are budded off the end of the ITs before the plant-derived cell wall surrounds the IT. The endocytotic budding process results in the releasing of bacteria into the plant cell surrounded by a plant-made membrane (Ardourel et al., 1994). Then, bacteria differentiate and express nitrogen fixation-required genes (Fischer, 1994; Mesa et al., 2008). Proteome studies of nodule environment have explained that it is an extraordinary case of an interaction that includes specific nutrient uptake systems (Day et al., 2001; Djordjevic, 2004), a specialized electron transport chain (Ekman et al., 2008; Mastronunzio and Benson, 2010), and specific modifications by bacteria to their lipopolysaccharide surface (Lee et al., 2008). Thus, the symbiotic environment forms a unique type of extracellular biology interaction within the plant cells.

The root hairs provide a brilliant position for microbial development. To this aim, it seems that rhizobia have various mechanisms to affix to roots including surface polysaccharides and secretory/surface proteins (Matthysse and Kijne, 1998; Rodriguez-Navarro et al., 2007). Many factors such as pH, nitrogen concentrations (Jamet et al., 2007; Cardenas et al., 2008; Chang et al., 2009), and specific growth conditions (Khatoon et al., 2012) can affect on this contribution of different components. Santos et al. (2000) and Vargas Mdel et al. (2003) have revealed that several enzymes such as catalases and superoxide dismutases help rhizobia to survive at the oxidative stress. It has been proposed that plant-produced ROS are involved in cross-linking of glycoproteins in the matrix of the ITs (Brewin, 2004). Hence, the capability of bacteria to deal with extracellular oxidative stress, throughout the symbiosis, is obviously important.

ATTACHMENT AND SECRETED PROTEINS

Secreted proteins are essential for attachment. For example, the role of rhicadhesin in attachment to root hairs has been described by Smit et al. (1992) and Laus et al. (2006). Rhizobium-adhering proteins (RAPs), which are encoded by the *prsD* and *prsE* genes (Russo et al., 2006), are secreted across the inner and outer membranes by means of a Type I secretion system. Different rhizobia have the potential to secrete proteins into the periplasm via the general export pathway and the twin-arginine translocation (TAT) secretion system and also type I, III, IV, and VI secretion systems that can manipulate the symbiosis (Deakin and Broughton, 2009; **Figure 1**; **Table 1**).

Many proteins of a typical N-terminal transit peptide are secreted via the general export pathway and many of these are expressed throughout infection and other steps of symbiosis. Cellulase (CelC2) is one of the particular symbiotic enzymes which is predicted to be exported through the general export pathway with a typical transit peptide (Downie, 2010). This cellulase can erode the non-crystalline cellulose in the cell wall of root hairs and is thought to allow penetration of rhizobia during the initial stage of infection in root hair curls (Robledo et al., 2008). Proteomics-based studies have demonstrated that proteins pertain to the TAT pathway are secreted in their folded form, some containing cofactors, and have a signal peptide usually containing a distinctive pair of consecutive twin arginine residues in the motif RRXFF, where X is any residue and F a hydrophobic residue (Antelmann et al., 2001; Bendtsen et al., 2005).

TYPE I, III, IV, V, and VI SECRETION PATHWAYS

Proteins secreted via type I, III, IV, V, and VI secretion pathways, which translocated across the inner and outer membranes without a periplasmic intermediate stage (Hueck, 1998; Koronakis et al., 2004), are derived from the bacterial flagellar secretion system and have evolved into a mechanism that can deliver proteins from the bacterial cytoplasm into the cytoplasm of eukaryotic cells (Saier, 2004). Secreted proteins insert into the outer membrane,

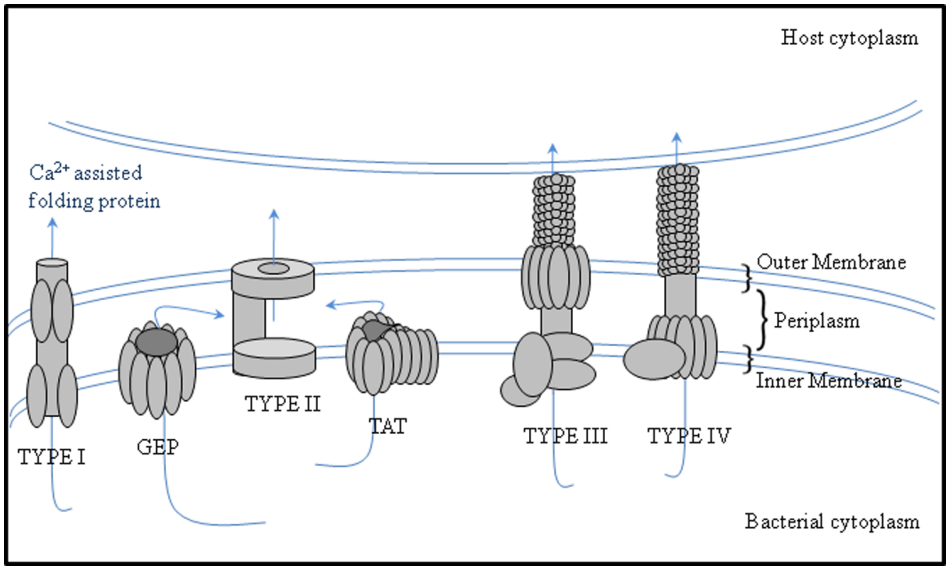

FIGURE 1 | Protein secretion systems secreting proteins to the periplasm or with periplasmic substrate intermediates in bacteria.
The schematic image of secretion systems in Gram-negative bacteria, which are involved in protein transport to the periplasm, are shown. Type I secretion systems translocate proteins from the periplasm, whereas Type II secretion systems translocate proteins from the periplasm and are thus dependent on the GEP or TAT pathways.

where it catalyses the translocation of the N-terminal region into the extracellular medium (Henderson et al., 2004) and mediates the translocation of proteins across the bacterial envelope (Bingle et al., 2008).

A cation selective channel protein, which is present in some rhizobia and can extend the ability to nodulation, is a calcium-binding protein called NodO (Economou et al., 1990; Sutton et al., 1994). NodO was found to insert into liposomes and form cation-selective channels in lipid bilayers (Miwa et al., 2006), and could therefore enhance the calcium spiking that is observed in root hairs upon Nod factor binding. Three possible roles for NodO have been suggested. The NodO protein may amplify the perceived Nod factor signal, facilitate Nod factor uptake by the host, or bypass the host's Nod factor receptor altogether (Sutton et al., 1994; Walker and Downie, 2000).

The type III secreted proteins as well as the effector proteins delivered into the plant cell have been called nodulation outer protein (Nop; Viprey et al., 1998; Marie et al., 2003). By comparing plant defense responses against bacteria, Bent and Mackey (2007) showed that the mutilation of symbiotic efficiency might lead to effector recognition by legumes and subsequently, increasing at defense response. And some other effectors have been identified such as NopJ, NopM, NopL, NopP, NopT and ImpK. Some of which are required for optimal nodulation in legumes (Bladergroen et al., 2003; Marie et al., 2003). NopL and NopP are phosphorylated by plant kinases (Bartsev et al., 2003, 2004; Ausmees et al., 2004; Skorpil et al., 2005). This process could point to a function in modulating host signaling pathways. Specifically for NopL, a role in the down-regulation of host plant defenses is proposed (Bartsev et al., 2004). NopT is a functional cysteine protease of the YopT family with a predicted myristoylation site (Dai et al., 2008). Some type III secretory proteins such as NopT have protease activities (Kambara et al., 2009), and some proteins such as NopJ and NopM have negative effects

Table 1 | Some important characteristics of protein translocation systems in Gram-negative bacteria.

	Secretion signal	Substrates	Reference
GEP	N-terminal signal peptide	Unfolded proteins	Paetzel et al. (2000)
TAT	N-terminal signal peptide with conserved twin-arginine motif	Fully folded proteins	Berks et al. (2003)
Type I	C-terminal signal peptide	Unfolded proteins	Wagner et al. (1983)
Type II	structural	Folded proteins or multimers	Pugsley (1993)
Type III	N-terminal signal	Unfolded proteins	Michiels et al. (1990)
Type IV	chaperone dependent	Folded or unfolded proteins or DNA	Berger and Christie (1994)
Type V	N-proximal secretion domain	Partly unfolded proteins	Henderson et al. (1998)

on the nodulation (Marie et al., 2003). YopJ family-like proteins acts as acetyl transferases and inactivate MAP kinases and contain protein–protein interacting leucine-rich repeats (Deakin and Broughton, 2009; Masson-Boivin et al., 2009). XopD and YopM are targeted to host cell nuclei and were interfere with the regulation of host proteins during infection (Skrzypek et al., 1998; Hotson et al., 2003). XopD, a cysteine protease, achieves this through hydrolysis of small ubiquitin-like modifier-conjugated proteins (Hotson et al., 2003), while YopM probably acts as a scaffold for recruiting and stimulating other proteins (McDonald et al., 2003).

Autotransporter proteins (type V) are secreted via the general export pathway into the periplasm using an N-terminal transit peptide and then a C-terminal domain inserts into the outer membrane, where it catalyses the translocation of the N-terminal region into the extracellular medium (Henderson et al., 2004). Recently, type VI secretion system has been recognized, mediating the translocation of proteins across the bacterial envelope (Mougous et al., 2006; Pukatzki et al., 2006). Proteins translocated by this system were identified and showed similarity to ribose-binding proteins from other bacteria (Filloux et al., 2008; Pukatzki et al., 2009). Some bacterial substrates and their roles were summarized in **Table 2**.

NODULINS AND LEGHEMOGLOBIN

Nodulins are organ-specific plant proteins induced during SNF. Nodulins play both metabolic and structural roles within infected and uninfected nodule cells. Nodulins are involved in the structure, development, maintenance, and general metabolism of nodule (Oaks and Hirel, 1985) and have been characterized from soybean (Lauridsen et al., 1993), pea (van de Wiel et al., 1990), and alfalfa (Hirsch et al., 1989). Although characterized prior to other nodulins, the leghemoglobins (Lbs) should be considered as major nodulins. Soybean contains four major leghemoglobins, Lba, Lbcl, Lbc2, and Lbc3, differing slightly in amino acid sequence (Lee and Verma, 1984).

The nodulin-24, a protein which is part of the peribacteroid membrane (Katinakis and Verma, 1985) and nodulin-23, along with Lb, are induced in the infected cells. The nodulin-35, the subunit of the tissue-specific uricase II (a tetrameric enzyme) and one of the most abundant proteins in soybean nodules (Takane et al., 1997), is found only in the specialized uninfected cells. Formation of the effective root nodule requires expression of symbiotic genes in the host plant and the micro symbiont (Verma et al., 1986) whose products are nodulins and bacteroidins, respectively. Nodulin-24 is a nodule-specific and nodule-enhanced pbm-bound protein (Cheon et al., 1994), which has a transport function. The genes coding for Lb, nodulin-23 and -24, whose transcription begin at about the same time, provide a rationale for the possible existence of 5′ *cis* sequences capable of binding *trans*-activator molecules. During nodule development, some products elaborated from the microsymbiont infection that may produce a *trans*-activator molecule. The presence of short sequences common to the flanking region of the genes concerned may provide the structural basis for induction. In view of a potential *cis*-receptor site upstream of nodulin genes, it is possible that these genes are positively regulated by a common transactivator molecule.

In indeterminating nodule-forming legumes, the leading nitrogen transport compounds are amides including glutamine and asparagine, whereas in determinating nodule-forming legumes, the major nitrogen products are ureides. This process occurs in plastids of both infected and uninfected nodule cell types (Streeter, 1991; Vance, 2008). Enzymes concerned may be "nodule-stimulated" proteins. A group of enzymes for the oxidation of purines into allantoin and allantoic acid are specifically induced during symbiosis. Xanthine dehydrogenase is a nodulin present in infected nodule cells (Montalbini et al., 1997; Todd et al., 2006) and can catalyze these purine oxidation steps in the infected nodule cell. The product is postulated to be transported to uninfected cells as uric acid. The oxidation of uric acid to allantoin is mediated by the oxygen-dependent enzyme uricase II (nodulin-35).

TRANSCRIPTION FACRORS

It is already obvious that TFs play fundamental roles in important processes in legumes and are involved in the control of mutualistic symbiosis such as SNF between plant root and rhizobia (Samac and Graham, 2007). More recently, TFs involved in the rhizobial infection process have been identified. Among them nodulation signaling pathway 1 (NSP1) and NSP2, GRAS-family proteins, are putative TFs that transduce the bacterial Nod factor signal and induce expression of plant nodulin genes and are required for nodule development (Kaló et al., 2005; Smit et al., 2005). In addition, other TFs crucial to the nodulation process were also identified by direct screening for nodulation-defective mutants. For example, NIN is the member of a novel family of putative TFs in higher plants, which are called the NIN-like family (Libault et al., 2009). Forward-genetics approaches have subsequently identified three other TFs (Nishimura et al., 2002; Mitra et al., 2004; Kaló et al., 2005; Smit et al., 2005) that are essential for nodule development, one of which is a Kruppel-like TFs of the C2H2 (Zn) family that was found to be crucial for differentiation of the nitrogen-fixing zone of nodules (Frugier et al., 2000). RNA interference (RNAi) method revealed a key role in nodule development for a member of the CCAAT-binding family of TFs (Combier et al., 2006).

QUORUM-SENSING

The rhizobia are excellent quorum-sensing model systems for such studies. The symbiotic relationships are the result of a complicated signaling network between the host and symbiont. Rhizobia populations regulate gene expression by autoinducers, diffusible signal molecules, which are interact specially with a receptor protein. Autoinducers production occurs at specific stages of development or in response to environmental changes. These diffusible signals commonly induce gene expression in response to bacterial cell density in symbiosis and often referred as quorum-sensing (Bassler, 2002). However, we are not quite familiar with the regulation of differentiation of bacterial and host–bacterium signal exchange. It is believed that nodule invasion requires the gathering of bacteria around the hair roots and the cell density of *Rhizobium* species should reach a threshold level (Caetano-Anollés and Gresshoff, 1991). Therefore, quorum-sensing probably plays a curial function in regulation of symbiosis. In addition, some rhizobial strains are able to synthesize rhizopines, opine-like compounds reminiscent of those produced by *Agrobacterium* species,

Table 2 | Examples of bacterial substrates and their roles.

Bacterial species	Substrate	Role	Secretion pathways
Agrobacterium rhizogenes	GALLS	Integration of T-DNA into plant genome	IV
Agrobacterium tumefaciens	VirD2	Nuclear localization and integration of T-DNA	IV
Bordetella pertussis	AB5 toxin	Interacts with α β γ heterotrimeric Gi/o proteins	IV
Bordetella spp.	SphB1	Proteolytic processing of secreted proteins	V
	CyaA	Leukotoxin, adenylate cyclase	I
E. coli	AIDA-1	Adherence	V
	AG43	Biofilm formation	V
	Tsh	Haemoglobinase activity	V
	EspP	Cytotoxic activity	V
	Vat	Vacuolating cytotoxin	V
	HlyA	Haemolysin	I
Erwinia spp.	PrtB/C	Metalloproteases	I
	DspA/E	Suppresses cell wall defense responses	III
	HrpW	Binds to pectate lyase	III
Haemophilus spp.	HMW1	Adhesin	V
	HxuA	Haem-haemopexin binding protein	V
	LspA1	Adhesin	IV
Helicobacter spp.	CagA	Leads to dephosphorylation of host cell proteins	IV
	Peptidoglycan	Induces NF-κB activity in gastric epithelial cells	IV
Legionella spp.	RalF	Exchange factor for ADP ribosylation factor	IV
	LidA	Docking of vesicles to the membrane of phagosome	IV
	DotA	Membrane pore in host	IV
	LepA	Alter exocytic pathway in protozoa	IV
	LepB	Alter exocytic pathway in protozoa	IV
	YlfA	Facilitate binding and fusion of *Legionella*-containing vacuole	IV
	YlfB	Facilitate binding and fusion of *Legionella*-containing vacuole	IV
	VipA	affects carboxypeptidase trafficking	IV
	VipD	interferes with multivesicular body formation	IV
	VipF	inhibits lysosomal protein trafficking	IV
Mesorhizobium spp.	Msi059	Deconjugates proteins stabilized by SUMOylation	IV
	Msi061	Target specific proteins for degradation	IV
Pasteurella spp.	LktA/C	Leukotoxin	I
Proteus spp.	HpmA	Cytolysin	V
Pseudomonas syringae	AvrPto	Inhibits hypersensitive response (HR)	III
	AvrRpt2	Cysteine protease	III
	AvrRpm1	Induces RIN4 phosphorylation	III
	HopPtoM	Suppresses salicylic acid-dependent callose deposition	III
	AvrPphC	Blocks AvrPphF-elicited HR	III
Rhizobium spp.	NopP/L	Suppress plant defense reactions	III
	PlyB	Glycanase processing extracellular polysaccharide	I
	NodO	Facilitates nodulation	I
	. Rzc-1	Bacteriocin activity	I
Salmonella spp.	SipA/C	Enhance actin polymerization and bundling	III
	SopE/E2	Activate G-binding proteins	III

(Continued)

Table 2 | Continued

Bacterial species	Substrate	Role	Secretion pathways
	SopB	Phosphatidylinositol phosphatase	III
Serratia marcescens	ShlA	Cytolysin	V
	SlaA S	Layer protein	I
	LipA	Lipase	I
	PrtA	Metalloprotease	I
	HasA	Haem-binding	I
Shigella flexneri	IcsA	Intracellular motility	V
	SigA	Cytopathic activity	V
Xanthomonas spp.	Hpa1/G	Elicits HR	III
	AvrXv4	Cleaves SUMO from sumoylated proteins	III
	XopD	Cleaves free and protein-bound SUMO	III
Yersinia spp.	YopH	Dephosphorylates Cas	III
	YopE	Activates signaling GTPases	III
	YopT	Cysteine protease	III
	YopO	Exhibits serine/threonine kinase activity	III
	YopJ	Cysteine protease	III
	YopM	Forms a complex with and activates kinases RSK1 and PRK2	III

Type I secretion is completely independent of the general export pathway (GEP) and transports proteins directly from the cytoplasm across both membranes to the extracellular space. Type II secretion systems translocate proteins from the periplasm and are thus dependent on the GEP or TAT pathways. Type III systems translocate proteins across both membranes using ATP. Type IV systems is capable of transporting both DNA and proteins. This system is used to introduce the T-DNA portion of the Ti plasmid into the plant host. Type V secretion or autotransporters, is a simple system which the secreted protein transports itself across the outer membrane after being transported to the periplasm by the GEP.

which are produced by the bacteroids and can be catabolized by the free-living associated rhizobia as a nutrient source (Murphy et al., 1987). These strains could putatively affect the dynamics of soil rhizobial populations.

In *R. leguminosarum* and *R. etli*, quorum-sensing probably is involved in restricting the number of nodules and in symbiosome development (Rosemeyer et al., 1998; Daniels et al., 2002; Wisniewski-Dye and Downie, 2002), and in *S. meliloti*, quorum-sensing controls the production of EPS II, an exopolysaccharide involved in the nodule invasion process (Marketon and González, 2002; Marketon et al., 2003). In different *Rhizobium* species, genes related to biosynthesis of exopolysaccharide, nodulation, and nitrogen fixation are located on one or more megaplasmids known as symbiotic (Sym) plasmids (Beynon et al., 1980; Banfalvi et al., 1985). Most of the nitrogen-fixing rhizobia regulate plasmid transfer via quorum-sensing systems, as in *A. tumefaciens*. While these systems do not seem to be essential for nodulation, they may play a role in rhizosphere survival (He et al., 2003). Although many aspects of the signal transduction are still an ambiguity, quorum-sensing has been intricate as an essential factor in the symbiotic process.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

A considerable branch of the genome is devoted to the synthesis of the various proteins and the regulation of the interaction with their complex environment. While remarkable progress in proteomic study of symbiosis has been made in model plants, a quite advancement in developing proteomic approaches in other crops has been reached. The biggest obstruction to these proteomic applications is the scarcity of well-annotated protein databases and sequences of proteins. Although some techniques such as *de novo* sequencing and proteogenomics recompense this paucity, there is still an urgent need to expand and curate plant protein databases. Many existing databases, including Soybean, *Medicago* and rice proteome database should be expanded and integrated in the future (Komatsu et al., 2004; Sun et al., 2009). In addition, most proteomic studies lead to protein identification and functional predictions yet the majority do not test their results using genetic approaches. A combination of proteomic analysis with genetics and other omics approaches would intensify the biological significance of many studies (Kint et al., 2010; Salavati et al., 2012a). Furthermore, transcriptomics and analysis of strains carrying multiple mutations will help in future research. A more systematic integration of interdependent techniques would provide valuable information and leads to better prediction and management of plant responses to symbiotic bacteria.

On the one hand, up to 20% of net photosynthetic production would be used for production and maintenance of root nodules (Pate and Givnish, 1986). Therefore, legume can invest in nodules if a decrease in net photosynthesis is compensated by the nitrogen fixation (Bethlenfalvay et al., 1978). On the other hand, there is no guarantee that all inoculated rhizobia strains will ensure a net benefit because rhizobial strains have been demonstrated to vary considerably in the advantages that they provide to the

legume (Thrall et al., 2007). If legumes could be able to discriminate between slightly and highly effective bacterial strains at the time of infection, there would be slight host carbohydrate loss to non-effective strains. Because, the nitrogen fixation begins a few days after inoculation, pre-infection recognition and exclusion of non-effective strains is inefficient.

Moreover, practical difficulties, such as high cost of performing field trails on Nod factor effects on nodulation, low stability of Nod factors in the soil due to quickly hydrolyzation in the rhizosphere by plant enzymes (Staehelin et al., 1994) and subsequently low biological activity of hydrolyzed derivatives (Heidstra et al., 1994) and mechanism of auto-regulation of nodulation (Caetano-Anollés and Gresshoff, 1991; Salavati et al., 2012a), lead to encountering the applied science to a new challenges which include discovering the bacterial and legume proteins, metabolites and their related genes effective on the signal production, perception, and transduction between partners. This proteomics strategy may open up an alternative perspective for improving symbiosis. Identification of genes and proteins and their related pathways may eventually lead to the production of new recombinant organisms, which have higher efficiency and ability to provide symbiosis and nitrogen fixation.

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Plant-bacterium interactions analyzed by proteomics

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The evolution of the plant immune response has resulted in a highly effective defense system that is able to resist potential attack by microbial pathogens. The primary immune response is referred to as pathogen associated molecular pattern (PAMP) triggered immunity and has evolved to recognize common features of microbial pathogens. In response to the delivery of pathogen effector proteins, plants acquired R proteins to fight against pathogen attack. R-dependent defense response is important in understanding the biochemical and cellular mechanisms and underlying these interactions will enable molecular and transgenic approaches for crops with increased biotic resistance. Proteomic analyses are particularly useful for understanding the mechanisms of host plant against the pathogen attack. Recent advances in the field of proteome analyses have initiated a new research area, i.e., the analysis of more complex microbial communities and their interaction with plant. Such areas hold great potential to elucidate, not only the interactions between bacteria and their host plants, but also of bacteria-bacteria interactions between different bacterial taxa, symbiotic, pathogenic bacteria, and commensal bacteria. During biotic stress, plant hormonal signaling pathways prioritizes defense over other cellular functions. Some plant pathogens take advantage of hormone dependent regulatory system by mimicking hormones that interfere with host immune responses to promote virulence (vir). In this review, it is discussed the cross talk that plays important role in response to pathogens attack with different infection strategies using proteomic approaches.

Keywords: pathogen associated molecular patter, pattern recognition receptors, effector triggered immunity, virulence, pathogenic bacteria, symbiotic bacteria, proteomics

INTRODUCTION

Unlike plant and animal cells, most bacteria are exposed to a constantly changing physical and chemical environment. Phylogenetic diversity of plant-associated bacteria (PAB) can categorize them in to commensals (acquire nutrients from the plant without damaging), mutualists (positively influence plant health), and pathogens (damage plant) (Newton et al., 2010). Notably pathogenic, commensals, or mutualists bacteria have developed strategies to interact with plants overlap, exceptionally modified physiology that accounts for individual need (Martin et al., 2003; Boller and Felix, 2009). Bacteria react to changes in their environment through changes in patterns of structural proteins, transport proteins, toxins, and enzymes, which adapt them to a particular habitat (Boller and Felix, 2009). Enzymes are either constitutive in nature (always produced by cells independently of the composition of the medium) or inducible (produced in cells in response to the end product of a pathway). Regulation of enzyme activity which is mainly operates to regulate biosynthetic pathways and catabolites repression is considered a form of positive control because it affects an increase in rates of transcription of proteins (Deutscher, 2008).

Plant immunity that recognize pathogens by membrane proteins is termed as pattern recognition receptors (PRRs), which recognize pathogen associated molecular pattern (PAMP) and is basis of plant innate immunity (Gomez-Gomez and Boller, 2000). PAMP recognition also results in plant systemic acquired resistance and production of resistance (R) proteins leading to effector triggered immunity (ETI), which is often accompanied by the hypersensitive response (HR), and programmed cell death (Jones and Dangl, 2006). Over the past 10 years, many R genes have been isolated that confer resistance to various pathogens including virus, bacteria, fungi, or nematodes (Martin et al., 2003). Based on predicted protein sequences, these R gene products are divided into intracellular protein kinases (Pto), proteins with an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic protein kinase region (e.g., *Xa21*), intracellular proteins containing a region of a LRRs and a nucleotide binding site (RPS2,

Abbreviations: PAMP, pathogen associated molecular pattern; PRRs, pattern recognition receptors; PAB, plant-associated bacteria; ETI, effector triggered immunity; R, resistance proteins; HR, hypersensitive response; EF, Elongation factor; T2SSs, T3SSs, T4SSs, T6SSs, type 2,3,4,6 secretion systems; LRR, leucine-rich repeat; EFR, elongation factor receptor; FLS2, flagellin sensing 2; vir, virulence; SA, salicylic acid; JA, jasmonic acid; MAP, mitogen-activated protein; 2-DE, two-dimensional polyacrylamide gel electrophoresis; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; LC, liquid chromatography; MS/MS, tandom mass spectrometry; SOD, superoxide dismutase; ROS, reactive oxygen species; hrp, hypersensitive reaction and pathogenicity gene; RuBisCO, ribulose-1, 5-bisphosphate carboxylase/oxygenase; PR, pathogenesis-related; GST, glutathione S-transferase; Hcp, hemolysin- coregulated proteins; OMVs, outer membrane vesicles; CPLL, hexapeptide ligand libraries.

RPM1), intracellular proteins containing a region of homology to the Toll/IL-1R proteins in addition to LRRs and a nucleotide binding site (e.g., N, L6, RPP5), and proteins with LRRs that appear to encode membrane-bound extracellular proteins (e.g., Cf-4, Cf-9) (Chisholm et al., 2006; Zhang et al., 2012).

Proteomic analyses have made possible the analysis of complex microbial communities, which had great potential to elucidate not only the interactions between bacteria and their host plants, but also of bacteria-bacteria interactions. Proteomic reference data sets were established for various PAB, via two-dimensional polyacrylamide gel electrophoresis (2-DE) gels, resulting in a few hundred identified proteins (Rosen et al., 2004; Chung et al., 2007), or multidimensional liquid chromatography-tandom mass spectrometry (LC-MS/MS) techniques resulting in the detection of more than 1000 proteins (Anderson et al., 2006; Bosch et al., 2008). Era is followed by gel free proteomics but quantitation procedures have to be optimized before the gel-based proteomics can be replaced by gel free procedures (Washburn et al., 2001).

Complete genome sequence of a *Xylella fastidiosa* is available which can be very helpful in genomics and proteomic studies of plant-bacterium interactions (Simpson et al., 2000; Bagnarol et al., 2007). More genomic data is needed for pathogenic and symbiotic bacteria to understand the molecular signaling pathways involved in plant-bacterium interactions. *P. syringae* and *Xanthomonas campestris* are important PAB on the grounds of agricultural importance and intensity of scientific research. Both are pathogenic on the model plant *Arabidopsis* (Jones et al., 2006a; Andrade et al., 2008). Pathogenic and mutualist PAB had been extensively studied (Rosen et al., 2004; Jacobs et al., 2012). The process of mutualism involved the significant change in the metabolism of both the mutualists and host, which involves a change in plant cell metabolism to support ATP synthesis and nitrogen fixation by the mutualist for nodule development (Delmotte et al., 2010). Transcriptomics data shows that pathogenic bacteria involve the hypersensitive reaction and pathogenicity (hrp) gene and different secretion systems (SS) for colonization and damaging host cells (Buttner and Bonas, 2010). In these review proteins of PAB bacteria have been compared. They typically exchange signals with their hosts and possess a range of specific adaptations for plant colonization. The importance of proteomics is explored to understand the molecular mechanism, by which bacteria adapt to live in association with plants for evolution of symbiosis and pathogenesis. This will opens up new research areas concerning protein-based plant-microbe communication and provides important information regarding the manipulation of gene expression of specific proteins with the purpose of modifying plant behavior related to compatible or incompatible interactions.

PAMP RECOGNITION BY PATTERN RECOGNITION RECEPTORS

PAMPs constitute the first layer of plant innate immunity, and lacking of its recognition can lead to enhanced disease susceptibility. PAMPs are ideal elicitors for "non-self" surveillance systems such as chitin, ergosterol, and transglutaminase from fungi, and/or lipopolysaccharides and flagellin from bacteria, stimulate plant encoded PAMP receptors (Chisholm et al., 2006). Intracellular responses associated with PAMP-triggered immunity (PTI) including rapid ion fluxes across the plasma membrane, mitogen-activated protein (MAP) kinase activation, production of reactive oxygen species (ROS), and rapid changes in gene expression and cell wall reinforcement. Suppression of PTI may be achieved by secretion of virulence (vir) effectors by the pathogens or by suppression of plant signaling. ETI is accompanied by production of R protein or HR, illustrating the dynamic co evolution between plants and pathogens (Jones and Dangl, 2006).

Flagellin, elongation factor (EF) Tu, peptidoglycan, lipopolysaccharide, and bacterial cold shock proteins are important PAMPS and plant responses induced by them are referred to as "basal" defenses (Newton et al., 2010). Upon recognition of highly conserved amino terminus of flagellin (flg22), flagellin sensing 2 (FLS2) induces a suite of defense responses, including MAP kinase signaling, transcriptional activation, and deposition of callose, a putative physical barrier at the site of infection (Gomez-Gomez et al., 1999). EF Tu potent bacterial PAMP in *Arabidopsis* and other members of the Brassicaceae family, serves as an adhesion factor at the bacterial surface, in addition to its primary role in translation (Boller and Felix, 2009). Asparate oxidase is required for PAMP-triggered RBOHD-dependent (responsible for stomatal closure) ROS burst and stomatal immunity against the *P. syringae* (Macho et al., 2012). The LRR receptor kinases, EF-Tu receptor and FLS2 are PRRs, contributing to disease resistance against the hemibiotrophic bacterium *P. syringae* (Roux et al., 2011).

The plant hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene, have emerged as key players in the signaling networks involved in plant immunity. Rhamnolipids are glycolipids produced by bacteria and are involved in surface motility and biofilm development and are considered as PAMPS. Ethylene is found to be involved in rhamnolipid-induced resistance to *H. arabidopsidis* and to *P. syringae* whereas JA is essential for the resistance to *B. cinerea*. SA participates in restriction of all bacterial and fungal pathogens, so involving in broadly rhamnolipid mediated immunity (Sanchez et al., 2012). PAMPS are sometimes succeeded and sometimes fails to induce PTI depending upon the type of compatible and non-compatible interactions. Flagellin is capable of suppressing HR via PTI induction during an incompatible interaction (Wei et al., 2012).

Type III secretion system (T3SSs) were essential components of two complex bacterial machineries: the flagellum, which drives cell motility and the non-flagellar T3SS (NF-T3SS), which delivers effectors into eukaryotic cells (Mudgett, 2005). *P. syringae* use T3SS to deliver up to 40 effector proteins into host cells, inhibiting basal host defense responses, such as HR (McCann and Guttman, 2008).

PAMP induced PTI serves as a primary plant defense response against microbial pathogens, with MAP kinase cascade downstream of PAMP receptors. LRR-RLKs including PSKR1 act as PTI against pathogenic bacteria, and plants expressing this gene show enhanced PAMP responses and less lesion formation after infection with the bacterial pathogen *P. syringae* via jasmonate signaling pathway (Mosher et al., 2013). Peptidoglycan, an important PAMP from *Staphylococcus aureus* results in PTI, such as

medium alkalinization, elevation of cytoplasmic calcium concentrations, nitric oxide, and camalexin production, and the post-translational induction of MAP kinase activities (Gust et al., 2007).

PAMP recognition also results in plant systemic acquired resistance and production of R proteins such as SUMM2 that becomes active when the MAP kinase cascade is disrupted by pathogens, leading to ETI (Zhang et al., 2012). In rice, the LRR-RK Xa21 confers resistance to *Xanthomonas oryzae* pv. oryzae strains carrying the Avr gene AvrXa21 (Song et al., 1995). AvrXa21 as a type I secreted sulfated peptide, is conserved among all *Xanthomonas* strains sequenced (P. Ronald, pers. communication), suggesting that *AvrXa21/Xa21* constitutes a PAMP/PRR perception system (Lee et al., 2008). Although many PAMPs recognized by plants have been described, number of known PRR and PTI is still in its infancy, constituting a highly active and competitive field of research.

PROTEOME ANALYSES OF PLANT ASSOCIATED BACTERIA

PAB either they are pathogenic or symbiotic bacteria adhere to plant surfaces, invade the intercellular space of the host tissue, counteract plant defense systems and acquire nutrients. However either there is establishment of a pathogenic interaction or mutualist relationship develops. Cell surface proteins such as adhesions, polysaccharides, lipopolysaccharides, and degradative enzymes enable the degradation of the plant cell wall and also result in basal plant defenses (Newton et al., 2010). Proteins of PAB are studied either in planta, by means of bacterial responses to selected biomolecule or plant extracts, synthetic media, or secretome analysis to study the vir factor of the bacterial pathogens (Guerreiro et al., 1997; Corbett et al., 2005; Gourion et al., 2006; Chung et al., 2007). All studies had helped to study plant-microbe interactions.

X. fastidiosa, whose genome was fully sequenced leads to a clearer understanding of the biology of phytopathogenic organism at both the genomic and proteome levels (Simpson et al., 2000). The cellular and secreted protein profiling of pathogenic bacteria *X. fastidiosa*, led to the identification of proteins involved in cellular adhesion systems, proteases, antioxidant, and toxins (Smolka et al., 2003). These proteins can be the candidates for understanding of molecular mechanism of disease cycle of citrus variegated chlorosis and Pierce's disease in grapevine caused by *X. fastidiosa*. In non-virulent strain of *E. chrysanthemi* (*opg* mutant), differential expression of protein is not restricted to the envelope, but affects general metabolism such as membrane lipid composition, protein folding, carbohydrate catabolism, and protein degradation (Bouchart et al., 2007). The secretome of *X. campestris* pv. *campestris* using 2-DE and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS resulted in the identification of 87 proteins known to be involved in degradative activities and important for the infection of susceptible plant hosts (Watt et al., 2005).

Diverse pathogenic responses are reported due to differences in induction of vir between closely related strains. Chung et al. (2007) reported that degradative enzymes and virulent secreted proteins were only seen in the pathogenic *X. campestris*. Moreover, HtrA was identified in the virulent *X. campestris* pv. campestris strain (Chung et al., 2007). Unique proteins involved in T3SSs activities and iron uptake were only consistently expressed in the virulent strain of *P. stewartii* (Bouchart et al., 2007; Wu et al., 2007). Proteins accumulated in T3SS cascade were related to iron uptake, motility, adhesion, metabolism, and transcriptional regulation (Wu et al., 2007). The siderophore-based iron uptake system is a common mechanism employed by gram-negative pathogenic bacteria.

Bacteria are known to react to a number of signaling molecules released by plants by specific gene expression. In *Rhizobium leguminosarum*, the proteins of cells were analyzed in the presence and absence of 7, 40-dihydroxyflavone. Proteins related to amino acid metabolism and transport, flagellin, energy, translation and structure were induced (Guerreiro et al., 1997; **Table 1**). In *Agrobacterium tumefaciens* acetosyringone is sensed by the virA/G two component systems and induces expression of the vir genes encoded on the Ti plasmid (McCullen and Binns, 2006). Vir proteins were reported to have T4SSs for the delivery of bacterial T-DNA into the host cell and to have molecular chaperone functions as an assembly factor (McCann and Guttman, 2008; Lai et al., 2006). Incubation of *A. tumefaciens* in the presence of cut root material led to the induction of ribosomal protein, stress related chaperones, ABC transporters and sugar binding proteins indicating the significance of protein modifications in the interactions of Agrobacteria with plants (Rosen et al., 2003). Addition of suberin to *Streptomyces scabies* induced 17 proteins linked to primary, secondary metabolism and stress related pathway (Lauzier et al., 2008). Expression of superoxide dismutase (SOD) was enhanced in *Frankia* strains and *Streptomyces coelicolor* with plant extracts (Langlois et al., 2003; Bagnarol et al., 2007). Addition of carbohydrates such as rhamnose and ferulic acid to medium induce plant phenolic compound (rhamno galacturonate lyase and esterase) in *D. dadantii* (Kazemi-Pour et al., 2004). Other identified proteins of *E. chrysanthemi* were cellulase, flagellin, pectinases, endopectate lyases, pectin acetylesterases, pectin methylesterase, and polygalacturonase (Kazemi-Pour et al., 2004). Proteins involved in signal transduction, translation, ribosomal proteins and biogenesis, inorganic ion, lipid, amino acid, energy, transport, and metabolism were induced by plant extracts (Langlois et al., 2003). These results suggest that root exudates provide additional carbon sources to the bacteria and that physiological adaptation are required for efficient bacterial growth in the presence of plants. Differential proteins involved in carbohydrates, lipids, purines, metabolism, transcription, coenzymes, chaperones and iron transport regulation, essential for nitrogen fixation, seem to be strain dependent (Dixon and Kahn, 2004).

Proteome analysis is very tricky when dealing with separation of bacteria from infected plants and additional steps are needed to avoid the changes in proteome map. Protocols had been proposed for the bacterial separation by density gradient centrifugation using percoll or sucrose gradients (Gourion et al., 2006; Nomura et al., 2010). Jacobs et al. (2012) discussed the transcriptomics profile of *R. solanacearum in vitro* and he discussed the importance of T3SS in vir cascade of *Ralsotonia* (45% regulated by HR and Hrp gene). Proteome analysis of pathogenic bacteria *X. campestris* pv. campestris in

Table 1 | Expression profiling of bacterial strains in differential medias/plant associated bacteria as pathogens, symbiotic or epiphytes.

Organism	Pathogen	Proteomic techniques	No. of IP[1]	Virulence factor, secretion systems, proteins expressed *in vivo* or under defined medias in different bacteria	References
Xanthomonas campestris pv. Campestris/parasite	*Brassica oleracea*, (cv. Coracao de boi) 4–6 days after inoculation (DAI)	2-DE, MALDI-TOF/TOF	21	Proteins from young leaves of susceptible *Brassica* cv infiltrated with *X. campestris*: aspartate semialdehyde dehydrogenase, elongation factor thermo unstable, phosphomannose isomerase, adenine triphosphate (ATP) synthase, ribosomal protein, chaperonin, phosphoglycerate kinase, and ATPase.	Andrade et al., 2008
Frankia sp. Strains M16467/symbiont	*Morella cerifera and Myrica gale*	2-DE, MALDI-TOF MS	50	With and without *M. cerifera, M. gale* seed phenolic extracts. Twenty proteins with increased abundance: signal transduction mechanisms, translation, ribosomal structure and biogenesis, chaperone heat shock protein, translation, amino acid and lipid transport, and metabolism. Thirty proteins with decreased abundance: Post-translational modification, chaperones, replication, transcription, translation, recombination, repair, energy production, conversion, lipid, inorganic ion, coenzyme, nucleotide, carbohydrate and secondary metabolite (transport and metabolism), cell wall biogenesis, and defense.	Bagnarol et al., 2007
Pseudomonas savastanoi pv. savastanoi/6 weeks after infection	*Olea europaea* subsp. europaea cvs Galega and Cordovil de Serpa	2-DE, MALDI-TOF MS	7	Proteins accumulated *in vivo* in *O. europaea* stems after infection by *P. savastanoi*: aconitate hydratase, tellurium resistance protein, enolase, hypothetical protein, and calcium dependent protein kinase.	Campos et al., 2009
X. campestris pv. *campestris* strain	*X. campestris* pv. *campestris* strain 11A/17	2-DE, MALDI-TOD MS	281	General protein accumulation in *X. campestris*: energy, metabolism, carbohydrate, lipid, protein and cofactors and vitamins metabolism, biosynthesis of secondary metabolites, transcription, translation, replication and repair, membrane transport, signal transduction, cell motility (22 higly abundant proteins of virulent vs. avirulent: aspartate semialdehyde dehydrogenase, cold shock protein, elongation factor, cellulase, L-isoaspartate protein carboxyl methyltransferase, malate dehydrogenase, 50S ribosomal protein, fumaryl acetoacetate hydrolase, peptidyl-prolyl *cis-trans* isomerase, 10 kDa chaperonin).	Chung et al., 2007
Bradyrhizobium Japonicum/Symbiont	*Glycine max*/21 DAI	LCMS/MS, LTQ-Orbitrap MS	3587 genes/proteins	Proteins involved in translation, post transcriptional regulation, nitrogenase complex, aspartate amino transferase, carbon metabolism, translation, and nucleic acid metabolism.	Delmotte et al., 2010
S. scabies	Wild type/*tatC mutant* strains	2-DE, MALDI-TOF TOF MS	73	Tat dep virulence (vir) factors: glycosyl hydrolase domain, putative alpha-L-fucosidase, ABC-type Fe^{3+} transport system, periplasmic component, glycosyl hydrolase domain, hydrolase of the α/β superfamily, lipoprotein,	Joshi et al., 2010

(Continued)

Table 1 | Continued

Organism	Pathogen	Proteomic techniques	No. of IP[1]	Virulence factor, secretion systems, proteins expressed *in vivo* or under defined medias in different bacteria	References
				spermidine/putrescine transporter peptide-binding protein, and rhamnosidase.	
Sinorhizobium meliloti/Symbiont	*Medicago truncatula* and *Melilotus alba*	2-DE, MALDI-TOF MS	420/700	Proteomes of the nodule bacteria compared to *S. meliloti in vitro*: transport proteins (ABC transporters, leucine and Iron binding protein), vitamin synthesis, stress-related processes, superoxide dismutase, betaine aldehyde dehydrogenase, heat shock protein, energy, GTP-binding proteins, oxidoreductase NAD protein, catalase, cell division, ribosomal protein, deoxyribonucleic acid transcription, translation, and central metabolism.	Djordjevic, 2004
Methylobacterium extorquens/epiphyte	*A. thaliana*	2-DE, LC MS/MS	45	PhytR regulated proteins: alcohol dehydrogenase, catalase, reactive oxygen species, stress proteins, lactoylglutathione lyase, dioxygenase, glutathione-dependent formaldehyde dehydrogenase, malyl lyase, malate dehydrogenase, glutathione-dependent formaldehyde dehydrogenase, haloacetate dehalogenase, transcription elongation factor, lipid metabolism, propionyl-CoA carboxylase, and dehydrogenases reductases.	Gourion et al., 2006
Rhizobium. *R. leguminosarum* bv. *trifolii* strain ANU843/symbiont	flavonoid 7,4 dihydroxyflavone (released due to legume nodulation)	2-DE, Protein sequencer	12	*R. leguminosarum* grown in presence and absence of 7,4-dihydroxyflavone: amino acid metabolism and transport, flagellin, protein translation and structure, energy, DnaK, NodE, and NodB.	Guerreiro et al., 1997
Azoarcus sp. strain BH72/endophyte	PilR mutant (necessary for endophytic growth)	2-DE, MALDI-TOF MS/MS, LC-MS/MS	785/30 abundant	PilR mutant proteins: amino acid and energy metabolism, chaperones, iron metabolism and storage, ATP synthase, ABC transporter, heat shock protein, pyridoxal phosphate, DNA, RNA polymerase, *S*-adenosyl-L-homocysteine hydrolase, fumarate hydratase, and ATP-dependent protease.	Hauberg et al., 2010
Burkholderia glumae (grain and seedling rot in rice, bacterial wilt in many crops)	*Under hrpB expression (induced after attack to plant)*	2-DE, ESI-MS/MS	34 secretory/12 cytoplasmic	Induction of type III secretion system (T3SS), acetylglutamate kinase, ribosomal protein, transcription elongation factor, Acetyl-CoA biotin carboxyl carrier protein, chaperone protein DnaK, lipoprotein, ATP synthase, phenylalanyl-tRNA synthase, and alkyl hydroperoxide reductase.	Kang et al., 2008
Rhizobium leguminosarum Biovar viciae (symbiont)	*Pisum sativum* and *Vicia cracca*	Microarray		Genes expressed related to tricarboxylic acid cycle, Succinate, pyruvate/inositol catabolism, α aminobutyrate metabolism, regulators, exported and cell surface molecules, multi drug exporters, and heat and cold shock proteins (early induced) *fix* genes, *nif* (late induced).	Karunakaran et al., 2009
Erwinia chrysanthemi (soft rot)	*Chrysanthemum* leaves extract	2-DE, MALDI-TOF MS	25	Endopectate lyases, pectin acetyl esterases, pectin methylesterase, poly galacturonase, flagellin, and elongation factor.	Kazemi-Pour et al., 2004

(Continued)

Table 1 | Continued

Organism	Pathogen	Proteomic techniques	No. of IP[1]	Virulence factor, secretion systems, proteins expressed *in vivo* or under defined medias in different bacteria	References
Agrobacterium tumefaciens	200 μM acetosyringone (As)	2-DE, MALDI-Q-TOF MS	11	As induced vir proteins, type IV secretion system (T4SS), newly As-induced proteins regulated by the virA/virG, an unknown protein Y4mC, and heat shock protein.	Lai et al., 2006
Streptomyces coelicolor	*Lemna minor fronds*	2-DE, MALDI-TOF MS	31	Differential proteins of *S. coelicolor* with and without *L. minor* fronds in minimal medium: proteins related to energy, metabolism, protein synthesis, proteins involved in the acquisition of carbon, stress-induced (chaperonin, ATP-GTP binding protein, tellurite resistance protein, and Fe superoxide dismutase).	Langlois et al., 2003
S. meliloti strain 2011	*Medicago truncatula*/3–6 D for drought	LC MS/MS	377	Proteins accumulated in *M. truncatula in vivo* with *S. meliloti* under drought: protein synthesis/degradation, stress proteins, RNA regulation, secondary metabolism, signaling, amino acid carbohydrate, nucleic acid/hormone metabolism, glycolysis/tricarboxylic acid cycle.	Larrainzar et al., 2007
Streptomyces scabies	potato suberin, lipidic plant polymer	2-DE, Protein sequencer MS/MS	19	Differential proteins in *S. scabies* in minimal media with and without 0.2% potato suberin: proteins with increased abundance in response to suberin (Nucleotide and amino acid metabolism, glycolysis/tricarboxylic acid cycle enzymes, chaperonin, phenylacetaldehyde dehydrogenase, fructose-bisphosphate aldolase, triose phosphate isomerase, ABC transporter, ATPase subunit, lipoprotein, and serine hydroxymethyl transferase).	Lauzier et al., 2008
Frankia sp. strain CcI3 (Symbiont)	*Casuarina cunninghamiana and C. glauca/Alnus incana* and *Elaeagnus angustifolia*	2-DE, MS/MS, LC MS/MS	73/53	*Frankia strains* in root nodules of *Alnus incana* and *Elaeagnus angustifolia* (cell wall/growth enzymes, solute-binding proteins (amino acids, peptides, inorganic ions) (ABC transporter, molybdate binding proteins, Leu/Ile/Val/Glu/Thr-binding protein precursor, hydrolytic enzymes, proteins involved in cell processes (DNA polymerase, signal transduction histidine, and T2SS protein).	Mastronunzio et al., 2009
Pectobacterium atrosepticum (Pathogen in potato)	Potato stem/tubers extract	2-DE, MALDI-TOF MS	40	Abundant proteins in minimal medium with potato tuber extracts: (T3SS protein, pectin enzymes, vir protein Svx, flagellar hook associated protein, endo-polygalacturonase, dihydrolipoamide dehydrogenase, hexosaminidase, pectate lyase, fructose bisphosphate aldolase, ABC transporter, glyceraldehyde 3-phosphate dehydrogenase, and chaperones).	Mattinen et al., 2007
Ralstonia solanacearum UW551 (phylotype II) and GMI1000	*S. lycopersicum* cv. Bonny Best	Microarray	109 by HrpB	Enhancement of hrpB regulated genes via T3SS, sucrose uptake and catabolism via SCR ABC, sucrose dependent phosphoenol pyruvate-carbohydrate phosphotransferase, glycolysis enzymes, cell wall-degrading	Jacobs et al., 2012

(Continued)

Table 1 | Continued

Organism	Pathogen	Proteomic techniques	No. of IP[1]	Virulence factor, secretion systems, proteins expressed *in vivo* or under defined medias in different bacteria	References
				enzymes, exopolysaccharide, reactive oxygen species, inorganic, organic and aminoacid and nucleic acid metabolic enzymes, tricarboxylic acid cycle intermediates, and pentose phosphate pathway.	
B. japonicum USDA110 (Symbiont)	*G. max* L. Merrill cultivar Akishirome. 7–49 DAI	2-DE, MALDI-TOF MS	275	Proteins accumulation related to transcription, Nif and Fix proteins, translation, protein folding, and degradation, synthetic enzyme of the poly-beta-hydroxybutyrate, solute transporter, and elongation factor-thermo unstable.	Nomura et al., 2010
A. tumefaciens. C58 strain ATCC 33970	*Solanum lycopersicum* cv. Rutgers stem, roots	2-DE, MALDI-TOF MS	30	Augmentation of ABC transporter, ATPase protein, alcohol dehydrogenase, enoyl CoA hydratase/isomerase, aldehyde dehydrogenase, protein-L-isoaspartate O-methyltransferase, acetyl COA carboxylase, ribosomal proteins (chaperonin, hydrolases, and sugar-binding protein), and aldolase.	Rosen et al., 2003
B. japonicum USDA110	HM medium (Cole and Elkan, 1973)/root nodule residing *B. japonicum*	2-DE, MALDI-TOF MS	1200	Suppression of fatty acid, nucleic acid and cell surface synthesis, DNA metabolism-related proteins and proteolytic enzymes. Enhancement of translation, transcription related proteins, elongation factor, chaperones (heat shock protein, chaperonin), ATP synthase, DNA polymerase, and nitrogen metabolism proteins.	Sarma and Emerich, 2006
B. japonicum USDA110	*G. max*. cv. Williams 82. Nodule protein	2-DE, MALDI-TOF MS	180	Augmentation of proteins related to nitrogen, carbon metabolism, protein synthesis, scaffolding and degradation, cellular detoxification function (ATP synthetase, elongation factor ribosomal protein, chaperonin, heat shock protein), stress regulation, signaling communication. Decline of fatty acid and nucleic acid metabolism, solute transport (ABC transporter) proteins, protein synthesis, scaffolding and degradation, cellular detoxification, stress regulation and signaling communication.	Sarma and Emerich, 2005
B. japonicum	*G. max, Vigna unguiculata* and *Macroptilium atropurpureum*	LC-MS/MS	2000	Accumulation of housekeeping proteins, ribosomal proteins, ABC-type transporter sulfonate-binding protein, enoyl-CoA hydratase, transketolase, hydroxyphenylpyruvate dioxygenase, nitropropane dioxygenase.	Koch et al., 2010
Xylella fastidiosa strain 9a5c	Brazilian sweet orange	2-DE, MALDI-TOF MS	30	Synthesis of aconitate hydratase, DNAk protein, dihydrolipoamide dehydrogenase, lipase/esterase, 30S, 50S ribosomal protein, inosine-5′-monophosphate dehydrogenase, heat shock protein, peptidyl-prolyl cis-trans isomerase, ATP synthase, aspartate-B-semialdehyde dehydrogenase,	Smolka et al., 2003

(Continued)

Table 1 | Continued

Organism	Pathogen	Proteomic techniques	No. of IP[1]	Virulence factor, secretion systems, proteins expressed *in vivo* or under defined medias in different bacteria	References
				alcohol dehydrogenase, fructose-bisphosphate aldolase, malate dehydrogenase, ABC transporter ATP-binding protein, chaperone, elongation factor, and RNA polymerase.	
Xanthomonas citri subsp. citri	TSE medium (with sucrose and glutamic acid) induce pathogenesity	2-DE, LC MS/MS	1702	Expression of Hrp gene dependent T3SS enzymes, tricarboxylic acid cycle, glycolysis/gluconeogenesis, pentose phosphate pathway, other sugar metabolism, urea cycle, pyrimidine and purine biosynthesis, fatty acid synthesis and degradation pathways, polyamine biosynthesis, DNA, RNA, protein metabolism, initiation, elongation, transcription, translation factors, cell structure and function, division, transport, pathogenesis and vir.	Soares et al., 2010
X. campestris pv. *campestris* B100		2-DE, MALDI-TOF MS	87	Accumulation of outer membrane proteins with signal peptide, ATP synthase beta chain, 30s ribosomal protein, metabolic proteins, protein maintenance and folding (chaperonin, DNA k), and degradive enzymes (cellulase, lipase).	Watt et al., 2005
Pantoea stewartii subsp. stewartii (Pnss) vir/P. stewartii subsp. indologenes (Pnsi) avir	Stewart's bacterial wilt and leaf blight of maize and sweet corn	2-DE, LC MS/MS, MALDI-TOF MS	21	Proteins accumulation in virulent verses avirulent strain: siderophore-based iron uptake system, motility, adhesion, and biofilm formation (flagellin B homolog), pilus adhesion, transdolase, secretory protein (T3SS protein, HrcJ, 60 kDa chaperonin), mannosyl transferase, transdolase, LysR, transcriptional activator belonging to the AraC family, ABC transporter.	Wu et al., 2007
A. tumefaciens		LC MS/MS	12	Enhancement of T4SS secretome proteins, virB, hemolysin- coregulated protein, periplasmic binding protein, amino acid-binding periplasmic protein, hemin-binding lipoprotein, and dipeptide protein.	Wu et al., 2008
S. meliloti mutants 2011-3.4 and 1021Δ*hfq*	Alfalfa	2-DE, MALDI-TOF MS	33	Proteins lower in abundance were sugar transporters, enzymes of central carbon metabolism, glycine betaine, electron transport chain, iron, sugar catabolism, biosynthesis of aminoacids, vitamins, purines and pyrimidines, chaperonin, heat shock protein, and ABC transporter. Proteins augmented were metabolism of nitrogen sources (mainly amino acids), glycine cleavage system, metabolic enzymes such as ornithine cyclodeaminase, arginase, adenosylhomocysteinase, and phosphoenol pyruvate carboxykinase.	Torres-Quesada et al., 2010

[1] *IP: Number of identified protein.*

Abbreviations: DAI, days after inoculation; ATP, adenine triphosphate; As, Acetosyringone; vir, virulence; T3SS, Type III secretion system; T4SS, Type IV secretion system; 2-DE, two-dimensional polyacrylamide gel electrophoresis; MS, Mass spectrometry; MS/MS, Tandom Mass spectrometry; MALDI-TOF, Matrix-assisted laser desorption/ionization time of flight; LC, liquid chromatography.

association with *B. oleracea* and *Pseudomonas savastanoi* pv. savastanoi resulted in comprehensive expression analysis including stress and metabolic proteins (Andrade et al., 2008; Campos et al., 2009). Increased levels of proteins involved in xanthan biosynthesis, stress response, and the metabolism were induced in *X. campestris* in planta conditions compared with *in vitro* grown cells (Andrade et al., 2008). Chaperonin is reported to be involved in stress responses and EF, which acts as an important PTI in the plants, is the key component of the translational machinery of bacteria. Xanthan is an extracellular polysaccharide probably responsible for disease symptom in planta growth via mucoid appearance of the bacterial colonies and wilting of host plants by blocking the water flow in xylem vessels (Buttner and Bonas, 2010).

The differential proteome analysis of in planta and *in vitro* grown cells of *Methylobacterium extorquens* resulted in the identification of 45 metabolic proteins and proteins involved in stress response such as the extracellular protease, SOD, catalases, and the DNA protection protein (Gourion et al., 2006). The protein analysis of cyanobacteria living in symbiosis revealed several adaptations to a symbiotic lifestyle, including an increase in proteins involved in energy production and nitrogen fixation. On the other hand, proteins involved in photosynthesis were decreased, pointing toward a heterotrophic lifestyle under symbiotic conditions (Dixon and Kahn, 2004). The general proteome analysis of bacteroids is compared with *in vitro* grown cells in order to identify nodule specific adaptations, over time or when plants were exposed to drought stress (Delmotte et al., 2010; Nomura et al., 2010). ABC-type transporters was present in nodule bacteria for transport of amino acids and inorganic ions along with proteins involved in vitamin synthesis, fatty acid, nucleic acid, cell surface synthesis, and stress-related processes (Sarma and Emerich, 2006). Integrated proteomics and transcriptomics data for *B. japonicum* bacteroids resulted in 2315 proteins involved in carbon and nitrogen metabolism, including a complete set of tricarboxylic acid cycle enzymes, gluconeogenesis and pentose phosphate pathway enzymes, along with other proteins important in symbiosis. Amino acids (Asn, Gln, Pro), organic acids (threonic acid), sugars (Rib, maltose), and polyols (mannitol) were reported to be more abundant in symbiotic roots (Delmotte et al., 2010). In planta studies is very effective to study the interactions between plant and bacteria.

GENE REGULATION IN SYMBIOTIC BACTERIA

An effective nitrogen-fixing symbiosis establishes as a result of a complex molecular interplay between both partners that involves bacteria-plant signal exchanges and intricate signaling processes (Dixon and Kahn, 2004). Rhizobia start as epiphyte attaches to the root hair, penetrate through the epidermis, divide within the plant-derived infection thread and then invade the cortical cells. On the other hand, in response to the bacterial infection, the plant cortical cells form a new organ, the nodule, where the bacteria differentiate into bacteroids and commence nitrogen fixation. An increased abundance of proteins involved in protein synthesis and degradation was observed during the early stage of nodule development, which may be explained by a restructuring of the proteome to attain a symbiotic lifestyle. Transformation of a free-living bacterium to a nitrogen-fixing endosymbiont results in significant physiological and developmental changes in the rhizobia, including the expression of nif and fix genes, which encode the proteins involved in the nitrogen fixation process in later stages of infection (Karunakaran et al., 2009; Nomura et al., 2010). To withstand changing environmental conditions, the bacteria must possess the ability to undergo specialized physiological adaptations. These response mechanisms are complex and remain largely unexplored. Comparative proteomic display of methylobacterium grown *in vitro* and in planta leads to identification of metabolic proteins and proteins involved in stress response such as the extracellular protease HtrA, SOD, catalases, and the DNA protection protein. In this study, also a key regulator, PhyR, was identified and shown to be essential for phyllosphere colonization (Gourion et al., 2006). These proteins seem to be important for the symbiotic relationship of microbe with plant.

Proteomic and transcriptomics approach was used to identify genes whose expression is regulated by the NifA-RpoN system in *Rhizobium etli* in symbiosis with *Phaseolus vulgaris* (Salazar et al., 2010; **Figure 1**). Twenty four proteins associated with NifA and RpoN motifs were identified via differentially proteomic analysis of mutant and wild type *R. etli* strain CFN42. Transcriptomics analysis helps to identify proteins with isoelectric points or molecular weights outside the electrophoretic resolution range of the 2-DE. NifA is a regulator for nitrogen fixation genes in symbiotic diazotrophic bacteria, and controlled by the oxygen-responsive two-component FixL-FixJ/Redox-sensing system RegS-RegR (Dixon and Kahn, 2004). Two further studies support regulatory role of Hfq in symbiosis process in *S. meliloti* (Barra-Bily et al., 2010; Torres-Quesada et al., 2010). Hfq is suspected to contribute to control central metabolic pathways in free-living bacteria and affects survival and nitrogen fixation capacity of the symbionts in the nodules, probably via the regulation of nifA and fixK1/K2 (Torres-Quesada et al., 2010). Barra-Bily et al. (2010) found that oxidative stress proteins and proteins involved in RpoE regulation were down regulated in hfq mutant. High expression of TonB-dependent receptors involved in transport processes of various carbohydrates was reported in *Sphingomonas* (Delmotte et al., 2009).

Hauberg et al. (2010) reported the PilR regulated cascade differentially present in symbiont *Azoarcus* sp. strain BH72 as compared to its mutant. It may be explained by a restructuring of the proteome to attain a symbiotic lifestyle. Koch et al. (2010) discussed host adaptation of *B. japonicum* in nodules of soybean, cowpea or siratro. Transcriptomics and proteomics analyses resulted in the identification of seven unique gene products that were the house keeping gens, along with ABC transporter, substrate-binding protein and a monooxygenase. In planta studies impart PhyR regulation along with the role of T3SS in infection process. The infection process resulted in augmentation of enzymes involved in gluconeogenesis, glycolysis, pentose pathway, amino acid, nucleic acid, lipid metabolism along with stress related proteins.

Protein secretion of a symbiont (*Sinorhizobium meliloti*) and a pathogen (*P. syringae* DC3000) in the presence of root exudates from *A. thaliana* and *Medicago sativa* was studied. SOD is

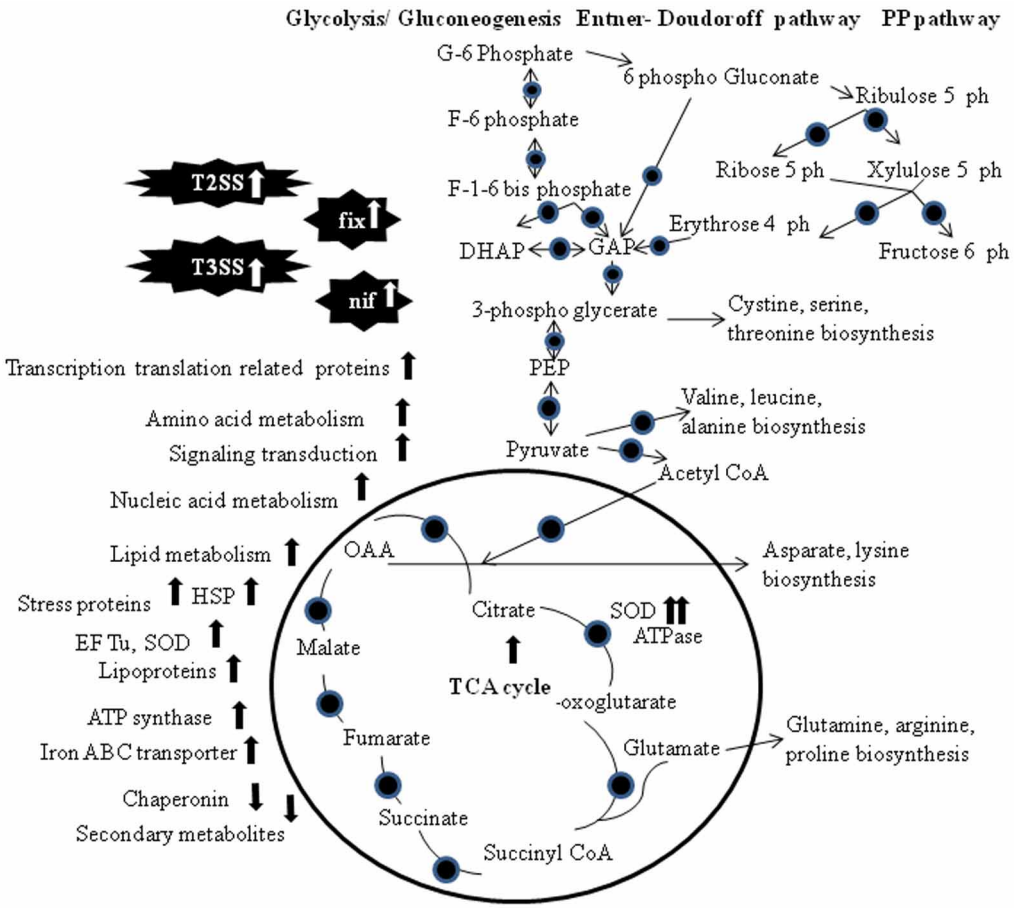

FIGURE 1 | Visualization of metabolic and proteomics pathways that may supply symbionts with energy under symbiotic conditions. The TCA cycle, gluconeogenesis/glycolysis, Entner–Doudoroff pathway and the Pentose Phosphate pathway are shown. Circles indicate proteins that have been detected at the transcriptomics and/or proteomics level. Abbreviations: PP, Pentose Phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; G, glucose; F, fructose; EF-Tu, elongation factor thermo unstable; SOD, superoxide dismutase; HSP, heat shock protein; T2SS, type II secretion system; T3SS, type III secretion system.

known to be necessary for symbiotic properties in *S. meliloti* and had higher expression in interaction with *M. sativa* (De-la-Pena et al., 2008). Extracellular proteins of the nitrogen-fixing symbiont *Frankia* were analyzed by dissecting nodules of infected cortical cells (Mastronunzio et al., 2009). The secreted proteins within the pool of identified proteins were recognized based on their export signal peptides by *in silico* prediction. Proteins detected in the secretome of three different *Frankia* strains were mainly solute-binding proteins, underlining the importance to acquire nutrients during symbiosis.

VIRULENCE FACTORS AND SECRETIONS SYSTEMS (SS) INVOLVED IN PLANT INFECTION

The genes encoding vir and symbiosis determinants are carefully regulated in order to prevent dissipative or premature expression. The expression of these genes is regulated by transcriptional regulators, which are part of regulatory systems such as the quorum sensing system and respond to different extracellular stimuli for instance, nutrients, oxygen levels, presence of plant-derived molecules or population density (Buttner and Bonas, 2010). The concerted action of different regulatory systems can vary substantially, even between closely related strains (Seo et al., 2008). A major task in understanding the regulation of gene expression is the identification of genes that are under the control of distinct regulators. Usually, transcriptomics studies are performed to address this question; however, proteomic analyses are also used, in particular when the aim is the evaluation of the impact on secreted proteins.

The establishment of a symbiotic or pathogenic association is largely dependent on the interaction between the microorganism and the host plant via secreted proteins. Effector proteins secreted by the pathogenic bacteria, either into the extracellular milieu or directly into the host cell cytosol target the host. The proposed biological roles and the mode of action of various effectors in plant pathogenicity have been reviewed (Mudgett, 2005). Combination of SS is involved in process of pathogenesis. In *Xanthomonas* spp six types of protein SS (I–VI) are involved in pathogenesis process (Bonemann et al., 2010). Effector proteins

interfere with the signaling cascades of susceptible hosts to counteract, for the plant innate immune responses, which are triggered by PAMPs (Mudgett, 2005). Proteomic studies were frequently applied to analyze the spectrum of proteins secreted by a particular system or to identify the system by which a certain protein is transferred.

The co-regulation of the synthesis of enzymes secreted via the T2SS and T3SS has been reported frequently (**Figures 1**, **2**). One reasonable explanation for this co-regulation is based on the observation that substrates that are secreted via the T2SS are not only associated with bacterial vir but can also induce plant defense responses. The concurrent induction of the T2SS and the T3SS probably allows the pathogen to counteract basal plant defense responses that are elicited by proteins released via the T2SS with the effector proteins transported by the T3SS (Jha et al., 2007). T3SS are used to inject different effector proteins, formerly known as avirulence proteins, directly into the host cell via an extracellular pilus that acts as channel (McCann and Guttman, 2008). T3SS is required for pathogenicity in host plants and for eliciting a rapid hypersensitive resistance response in non-host plants, and the genes encoding T3SS were named HR and pathogenicity (hrp) genes (Jacobs et al., 2012). hrp genes were identified in most Gram-negative plant pathogenic bacteria with the exception of *A. tumefaciens* and *Xylella fastidiosa* (Buttner and Bonas, 2010). Homologues of the hrp genes were also found in different rhizobia (Jones and Dangl, 2006). To identify the SS that is responsible for the transport of distinct vir factors, the secretome of a SS mutant strain and the wild-type strain is compared (Kazemi-Pour et al., 2004; Watt et al., 2009). Joshi et al. (2010) discussed twin arginine transport (Tat) dependent translocation of vir factor in *S. scabies* under T3SSs. Tat-secreted vir proteins includes lipoproteins, ABC transporters, phospholipases/phosphoesterases, β-lactamase, and proteins involved in Fe homeostasis and Tat dep vir factors (**Table 1**). T3SS-secreted proteins responsible for vir process usually silenced in plant-pathogenic bacteria and were only induced during the plant

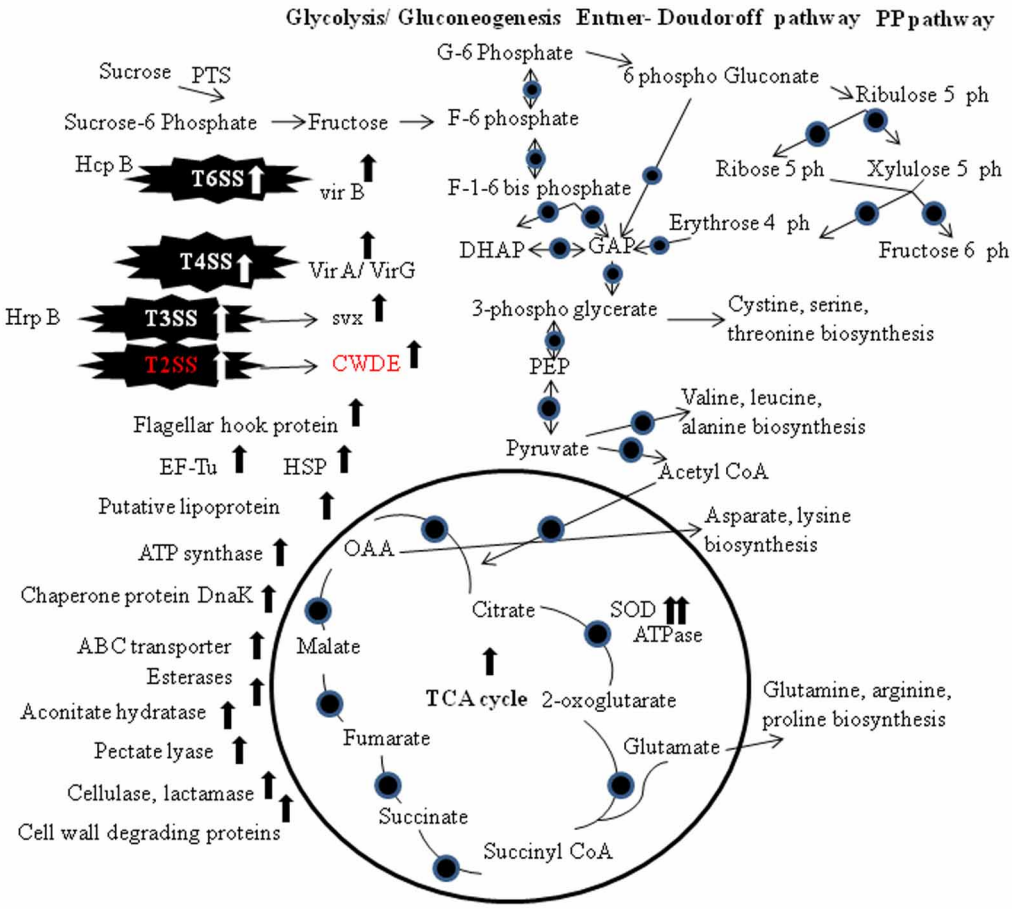

FIGURE 2 | Biochemical pathways of the pathogen secretion system and proteins expression. The TCA cycle, gluconeogenesis/glycolysis, Entner–Doudoroff pathway and the Pentose Phosphate pathway are shown. Circles indicate proteins that have been detected at the transcriptomics and/or proteomics level. Abbreviations: PP, Pentose Phosphate; PTS, phosphoenol pyruvate-carbohydrate phosphotransferase system; G, glucose; F, fructose; PEP, phosphoenol pyruvate; TCA, tricarboxylic acid; OAA, oxaloacetate; T6SS, type VI secretion system; T4SS, type IV secretion system; T3SS, type III secretion system; T2SS, type II secretion system; EF-Tu, elongation factor thermo unstable; SOD, superoxide dismutase; HSP, heat shock protein; CWDE, cell wall degrading enzymes.

infection process (Buttner and Bonas, 2002). Another vir factor Svx (secreted vir factor from *Xanthomonas*), in a secrotomic study of *P. atrosepticum*, was analyzed (Corbett et al., 2005).

Addition of galacturonate, pectin or plant extract to the medium resulted in the induction of *vir* factors in Enterobacteriaceae (Kazemi-Pour et al., 2004; Mattinen et al., 2007). In *Xanthomonas* secretome studies a defined medium with fructose, sucrose and casamino acids was found to induce T3SS gene (Wengelnik et al., 1996; Yamazaki et al., 2008). To study the secretome of *A. tumefaciens*, acetosyringone was added to induce VirB1 (Wu et al., 2008). *P. atrosepticum* in the presence of potato tuber extract, induce vir factors such as pectic enzymes, proteinase, and the avirulence protein homolog Svx (Mattinen et al., 2007). Likewise, culturing *X. campestris* pv. campestris in the presence of *B. oleracea* led to the induction of a nuclease, a ribonuclease, peptidases, proteases and cell-wall-degrading enzymes (Watt et al., 2009). T3SS was induced by addition of flavonoid (genistein) to the medium, resulted in secretion of the nodule outer proteins (Nops), which is involved in the host-range determination (Jones and Dangl, 2006).

Protein secretion into the extracellular space or other space was mediated by outer membrane vesicles (OMVs) in some bacterial strains. OMVs are an ideal structure to transport hydrophobic compounds like membrane proteins into host cells or quorum signals to neighboring cells (Wai et al., 2003; Mashburn and Whiteley, 2005). OMVs of gram-negative bacteria contain outer membrane proteins, periplasmic proteins, lipopolysaccharides, phospholipids, DNA, toxins, and other factors associated with vir (Kadurugamuwa and Beveridge, 1997; Mashburn and Whiteley, 2005). They are known to be constantly liberated from the outer membrane and are thus a likely source of outer membrane proteins in the culture supernatant (Kadurugamuwa and Beveridge, 1997). In the presence of vir factor inducing medium, two major protein groups were detected: outer membrane proteins and vir -associated proteins. The latter group included proteins of the T3SS, effector proteins, two cell wall-degrading enzymes, a cellulase, and a xylosidase.

Several periplasmic proteins were detected in the OMVs, which may be entrapped in the vesicle lumen during their release from the outer membrane. One of the identified vir proteins is HrpF, the putative translocon of the T3SS that is proposed to be inserted into the host membrane and serves as attachment site for the T3SS conduit. It is currently assumed that this protein is transported via the T3SS and then pushed into the host membrane (Buttner and Bonas, 2002). However, since OMVs of mammalian pathogens have been reported to deliver vir -factors into host membranes by fusion (Wai et al., 2003), it can be suspected that HrpF is inserted into the plant membrane by fusion of OMVs carrying HrpF.

Proteomic studies confirmed the transcriptional activation of vir factor (Hrp) via T2SS in *Burkholderia glumae* and *X. axonopodis* pv. citri (Kang et al., 2008; Yamazaki et al., 2008). Vir factor Svx was secreted via the T2SS in *P. atrosepticum* (Corbett et al., 2005). Protein secretion in the Gram-positive *S. scabies* via the twin arginine transport (Tat) pathway was analyzed. In Gram-negative bacteria the Tat system is part of the T2SS (Johnson et al., 2006). T2SS are present in many gram-negative proteobacteria, and toxins and extracellular enzymes such as proteases, lipases, and cell-wall-degrading enzymes were secreted via the T2SS (Jacobs et al., 2012). Components of the T2SS were identified as vir factors in the plant pathogenic bacteria *D. dadantii*, *Pectobacterium carotovorum*, *Xanthomonas* spp., and *Ralstonia solanacearum* (Buttner and Bonas, 2010). Comparison of the extracellular proteome of a tatC mutant and the wild type in combination with an *in silico* search for proteins harboring a secretion signal identified 73 predicted secretory proteins whose expression was reduced in the mutant. The tatC mutant strain was almost completely avirulent, indicating that the activity of this transport system is mandatory for vir. Hemolysin- coregulated proteins (Hcp) vir factor is structural component of a type T6SSs, components of the T4 bacteriophage tail tube and is required to puncture the host membrane in the context of phage infection (Bonemann et al., 2010). Hcp is transported via the T6SS in *A. tumefaciens* (Wu et al., 2008). Comparative proteome analysis with and without acetosyringone confirmed the induction of Vir proteins comprising the type IV secretion system for the delivery of bacterial T-DNA into the plant host cell. More induced proteins were identified, one of these is a molecular chaperone and functions as an assembly factor for the type IV secretion system (Lai et al., 2006; McCann and Guttman, 2008). The proteins that enable successful infection of plants are termed vir factors. These include cell surface proteins such as adhesins, polysaccharides, lipopolysaccharides, and degradative enzymes such as cellulases, pectate lyases, and proteases that enable the degradation of the plant cell wall under the action of T2SS (Jacobs et al., 2012; **Figure 2**).

PROTEOMICS ANALYSIS OF THE PLANTS AFFECTED BY BACTERIA

Inspite of lot of work on plant-microbe interactions, there are still lot of gaps in different proteomic studies of plants in response to bacterial infection. Here there is brief preview of some of research articles regarding plant responses to bacterial attack, which can be helpful to understand the plant microbe interactions (**Table 2**). Miao et al. (2008) reported that caffeoyl CoA 3-O-methyltransferase gene was down regulated in susceptible cultivars of tomato in response to inoculation with bacteria. Defense-related antioxidants such as pathogenesis-related (PR) -9 and metabolic enzymes were reported in *A. thaliana* in response to *P. syringae* (Jones et al., 2004, 2006a). Both of these groups of antioxidant enzymes were considered to have probable significant roles in the regulation of redox conditions within infected tissue. Many new techniques such as hexapeptide ligand libraries (CPLL such as proteominer) had been used to decrease the high abundant proteins for enrichment of low abundant protein. Frohlich et al. (2012) applied the CPLL in *A. thaliana* leaf proteins after infection with virulent *P. syringae*. 2-DE showed a decrease in high-abundance proteins and an enrichment of less abundant proteins in leaf samples. Mass spectrometric analyses of leaf extracts led to the identification of 312 bacterial proteins in infected *Arabidopsis* leaves.

Accumulation of free linolenic and benzoic acid or reduction in lauric acid was found to be important indicator of an

Table 2 | Proteomics analysis of plants in response to pathogenic/symbiotic bacteria.

Studied organism	Pathogen	Proteomic approach	No. of IP[1]	Identified proteins	References
A. thaliana	*P. syringae*	2-DE LC MS/MS	52	Abundant proteins (defense-related, Transcription, elongation factor, peroxiredoxin, arginase, lipase/acylhydrolase, PS11, sedoheptulose bisphosphatase, Protein folding and turnover, cytochrome, Cellular transport and ion homeostasis, decreased proteins, metabolism, and proteasome.	Jones et al., 2006a
A. thaliana	*P. syringae*	iTRAQ	5	Phosphoproteome changes 4 proteins (dehydrin, co-chaperone, heat shock protein, plastid-associated protein) and ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO LSU).	Jones et al., 2006b
A. thaliana	*P. syringae*	2-DE; LC MS/MS	2	Glutathione S-transferase (GST), peroxiredoxin.	Jones et al., 2004
O. sativa transgenic and/or inoculated with *Xanthomonas oryzae* pv. *oryzae*	*X. oryzae pv. oryzae*	2-DE MS/MS Protein sequencer	10	Accumulation of pathogenesis-related protein (PR) -5, superoxide dismutase, peroxiredoxin, glycine cleavage H protein, glyceraldehydes 3-phosphate dehydrogenase, triose phosphate isomerase, oxygen evolving complex is downregulated.	Mahmood et al., 2006
O. sativa	*X. oryzae pv. oryzae*	2-DE, MS/MS	20	Defense-related plasma membrane proteins with increased abundance are ATPase, phosphatase, hypersensitive response, prohibitin, zinc finger and C_2 domain protein, universal stress protein, heat shock protein, ascorbate peroxidase (APX), alcohol dehydrogenase, and quinone reductase.	Chen et al., 2007
O. sativa	*X. campestris* pv. Oryzicola	2-DE, MALDI-TOF MS	32	PR-1, PR-10, receptor-type protein kinase, ascorbate peroxidase, adenine triphosphate (ATP) synthase, RuBisCO LSU, ribonuclease, phospholipase, and GTP binding protein.	Li et al., 2012
L. hirsutum (leaflets)	*Cl. michiganensis* ssp. michiganensis	2-DE, ESI-MS /MS	47	Regulatory proteins accumulated: defense and stress related (PR-3, GST, APX, superoxide dismutase), regulatory proteins, protein synthesis and processing (chaperonin, elongation factor-thermo unstable), carbon metabolism: RuBisCO (LSU, small subunit, activase, epimerase, triose phosphate isomerase), metabolism [glycine cleavage system, oxygen evolving enhancer (OEE)], and ATP production [nucleotide diphosphate kinase (NDK), ATP synthase].	Coaker et al., 2004
M. truncatula, root	*S. meliloti*	2-DE, PMF	99	Proteins increased in abundance: defense and stress related (PR-9, -10, APX, superoxide dismutase), S-adenosyl methionine synthase, GST, elongation factor, NDK, protein disulfide isomerase (PDI), OEE, protein synthesis and degradation, isoflavone reductase, hormone dependent proteins, and metabolic proteins (ATP synthase, fructose bisphosphate aldolase).	Mathesius et al., 2003

(Continued)

Table 2 | Continued

Studied organism	Pathogen	Proteomic approach	No. of IP[1]	Identified proteins	References
S. lycopersicum	*P. solanacearum*	Protein sequencer	15	Proteins highly accumulated: protein destination and storage (60 kDa chaperonin, PDI, heat shock protein), protein synthesis, metabolism (RuBisCO activase, plastocyanin, glycine dehydrogenase, OEE), and defense (calgranulin, AMA).	Afroz et al., 2009
Rice (var. Co43)	*P. fluorescens* KH- 1 (PGPR)	2-DE, MS, LC MS/MS	23	Highly abundant proteins of energy metabolism, photosynthesis, protein degradation and antioxidation: GST, NDK, chaperone, thioredoxin, RuBisCO LSU, and proteosome	Kandasamy et al., 2009
Malus Domestica cv. Holsteiner Cox	*P. fluorescens*	SDS-PAGE, MS/MS	5	PR-2, -3, -4b, -5, -10, ribonuclease-like, endochitinase class III.	Kurkcuoglu et al., 2004
S. lycopersicum	*P. solanacearum*	Protein sequencer	15	Proteins with increased accumulation in response to JA and SA: defense (S-adenosyl methionine synthase, arginase, peroxiredoxin, threonine diaminase, polyphenol oxidase, leucine aminopeptidase), protein synthesis (PDI), protein destination and storage (60 kDa chaperonin), energy (glycine cleavage, ATP synthase), and RuBisCO LSU downregulated.	Afroz et al., 2009
G. max	*B. japonicum* (symbiotic root nodules)	GC MS, LC-QTOF-MS	166	Genes highly expressed: fatty acid, amino acid, carboxylic acid metabolism, disaccharides, isoflavonoids, and glucosinolate.	Brechenmacher et al., 2010

[1] *IP: Number of identified protein.*

Abbreviations: ATP, adenine triphosphate; GST, glutathione S-transferase; RuBisCO, ribulose-1, 5-bisphosphate carboxylase/oxygenase; LSU, large subunit; APX, ascorbate peroxidase; PR, pathogenesis-related protein; OEE, oxygen evolving enhancer; PDI, protein disulfide isomerase; NDK, nucleotide diphosphate kinase; 2-DE, two-dimensional polyacrylamide gel electrophoresis; MS, Mass spectrometry; MS/MS, Tandom Mass spectrometry; LC, liquid chromatography; GC, gas chromatography; MALDI-TOF, Matrix-assisted laser desorption/ionization time of flight.

active plant defense response in *G. max*. γ-aminobutyric acid, proline, and glutamine reduction resulted in *G. max* susceptibility after *Bradyrhizobium japonicum* inoculation (Brechenmacher et al., 2010). Transcriptomic and proteomic approaches identified numerous genes and proteins involved in carbon and nitrogen metabolism, plant defense responses, nutrient exchange, and signal transduction that are significantly regulated in *G. max* colonized by *B. japonicum* (Brechenmacher et al., 2010). Li et al. (2012) discussed importance of PR-1 and PR-10 in rice defense against *Xanthomonas campestris*. Components of PSII, mitochondrial permeability transition and cytoplasmic antioxidant enzymes were modified during R-gene-mediated HR. PR-9 and PR-5 were induced in response to *X. oryzae* pv. Oryzae in rice (Mahmood et al., 2006). Analyses clearly revealed that four defense-related proteins (PR-5, Probenazole-inducible protein, SOD and peroxiredoxin) were induced for both compatible and incompatible *X. oryzae* pv. Oryzae races, where PR-5 and probenazole-inducible protein were more rapid and showed higher induction in incompatible interactions and in the presence of JA. Furthermore, the sense PR-5 transgenic rice plants were more resistant than the susceptible vector control against *X. oryzae* pv. Oryzae (Mahmood et al., 2009).

Chen et al. (2007) analyzed proteins from rice plasma membrane to study the early defense responses involved in *Xa21* mediated resistance. *Xa21* is a rice receptor kinase, which is predicted to perceive the *X. oryzae* pv. oryzae signal at the cell surface, leading to the "gene-for-gene" resistance response (Song et al., 1995). Twenty proteins that were differentially expressed had potential functions in rice defense. Proteins expressed in partially resistant lines and a susceptible tomato species that are regulated in response to *C. michiganensis* ssp. michiganensis were identified 72 and 144 h post inoculation. Using 2-DE and MS, 26 differentially regulated tomato proteins were identified; 12 of which were directly related to defense such as PR-3, PR-9, and stress responses (Coaker et al., 2004). PR proteins seem to be good candidates to develop the incompatible interactions with bacteria along with SAR in plants. Proteomic analysis was also used to detect the responses of the model legume *M. truncatula* to the pathogenic bacterium *P. aeruginosa* (Mathesius et al., 2003). There is accumulation of 154 proteins, among which 21 are related to defense and stress responses. Molecular chaperones, protein related to defense, destination and storage were differentially expressed in resistant tomato cvs (Afroz et al., 2009). Apical membrane antigen was found to be the novel protein

expressed in susceptible cultivar in SA cascade (Afroz et al., 2010). Kandasamy et al. (2009) reported differential proteins in response to *P. fluorescens* in rice leaf sheath (**Table 2**). Along with protein degradation and antioxidation, photosynthetic proteins thioredoxin was upregulated. Defense proteins can be the other target protein for the disease resistance against bacterial pathogens. Tomato seed treatment with rhizobacteria *P. fluorescens* exhibited growth promotion along with protection from infection by *Fusarium oxysporum* (Ramamoorthy et al., 2002). PR-2, PR-3, and PR-5 were found to be induced in *P. fluorescens* treated plants challenged with *F. oxysporum*. Similarly, the expression of glutathione S-transferase (GST) is known to be involved in tagging toxic endogenous substrates with glutathione conjugation to transport toxic substrates into the vacuole through a glutathione pump (Ishikawa, 1992). GST is reported to be induced in response to *P. syringae* in *Arabidopsis*, and has an important role in plant defense from oxidative damages caused by various biotic or abiotic stresses such as heavy metal, wounding, ethylene, ozone, and pathogen attack (Marrs, 1996; Jones et al., 2004, 2006a,b).

Chloroplasts may be key players in plant defense, as the loss of integrity of PSII may lead to the HR-associated oxidative burst, thereby restricting pathogen growth. Proteins related to photosynthesis, defense, protein destination and storage had been down regulated in response to *P. syringae* infection in soybean (Zou et al., 2005). Proteins differentially expressed in response to *P. savastanoi* pv. savastanoi on *O. europaea* stems were related with photosynthesis and metabolism (Campos et al., 2009). Non-pathogenic bacteria (*P. putida*) had been reported to promote systemic resistance in different host plants as well. Stimulation of antifungal material (phytoalexin) and proteins in lipoxygenase pathway in tomato were found after infection with *P. putida* (Akram et al., 2008).

Holzmeister et al. (2011) used 2-D fluorescence difference gel electrophoresis technology to demonstrate the role of niric oxide in plant defense signaling. After infection of wild type and *Arabidopsis* mutant strain, it was found that in the infection by avirulent *P. syringae* there is accumulation of defense, redox as well as stress proteins, while in virulent infection only defense proteins were expressed. This imparts importance of stress proteins in plant defense signaling. Induction of defense enzymes involved in phenyl propanoid pathway and accumulation of phenolics and PR-proteins might have contributed to restriction of invasion of *F. oxysporum* f. sp. lycopersici in tomato roots. Induction of defense proteins and chemicals in tomato by *P. fluorescens* isolate against *F. oxysporum* was studied. Defense-related proteins such as PR-9, PR-2, PR-5, and 46 kDa chitinase increased in bacterized tomato root tissues (Ramamoorthy et al., 2002). Proteins related to photosynthesis, defense, destination, and storage are decreased as a result of bacterial infection followed by the induction of PR proteins in JA or SA pathway. Defense-related proteins, PR proteins and PSII destruction and repair system can be the candidates for improved resistance against bacterial infection in plants.

CONCLUSIONS

This review summarizes the use of proteomic approaches to understand the molecular and cellular processes that govern host responses such as PTI, PRR, and ETI. Detailed global comparison of response pathways using proteomics has allowed the identification of novel proteins whose biological role warrants in-depth biochemical and cellular elucidation. Finally understanding of biotic stress responses may identify promising novel targets for the development of cultivars with improved disease resistance. Various types of bacteria can trigger rapid responses in plant cell cultures and defense responses in intact plant tissues (Jones and Dangl, 2006). Flagellin and lipopolysaccharides have been identified as common bacterial determinants or PAMPs that act as elicitors of defense responses in plant cells (Felix et al., 1999). R genes that confer resistance to various phytopathogens are proposed to be part of a positive feedback loop to amplify the response triggered by PAMP perception. ETI is often accompanied by local cell death known as the HR illustrating the dynamic evolution between plants and pathogens (Martin et al., 2003; Jones and Dangl, 2006).

To fully elucidate microbial metabolism and its responses to environmental factors, it will be necessary to go beyond the information obtained from proteomic studies alone. There is need of integration of data resulting from the functional characterization and quantification of molecules representing from genes, transcripts and proteins to metabolites (Delmotte et al., 2010). First steps in this direction were made by setting up extensive proteomic reference databases and by compiling proteomic and transcriptomics data sets (Bosch et al., 2008; Koch et al., 2010). To profit from such analyses as best as possible, databases should be set up that combine and integrate the results obtained in different studies. With complete genome sequences of several host and pathogen partners now available, there are literally hundreds of candidate genes and proteins with potential applications in crop protection. Knowledge generated from research such as described in this special issue will open new avenues for the engineering of durable resistance to bacterial pathogens in plants. Identified proteins involved in plant tolerance to stresses could be used for development of disease resistant cultivars as well as these proteins can be used as markers for the identification of type of infection.

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Mass spectrometry based imaging techniques for spatially resolved analysis of molecules

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Higher plants are composed of a multitude of tissues with specific functions, reflected by distinct profiles for transcripts, proteins, and metabolites. Comprehensive analysis of metabolites and proteins has advanced tremendously within recent years, and this progress has been driven by the rapid development of sophisticated mass spectrometric techniques. In most of the current "omics"-studies, analysis is performed on whole organ or whole plant extracts, rendering to the loss of spatial information. Mass spectrometry imaging (MSI) techniques have opened a new avenue to obtain information on the spatial distribution of metabolites and of proteins. Pioneered in the field of medicine, the approaches are now applied to study the spatial profiles of molecules in plant systems. A range of different plant organs and tissues have been successfully analyzed by MSI, and patterns of various classes of metabolites from primary and secondary metabolism could be obtained. It can be envisaged that MSI approaches will substantially contribute to build spatially resolved biochemical networks.

Keywords: mass spectrometry, imaging, secondary metabolites, primary metabolism, proteins, peptides, metabolite distribution

BACKGROUND

Spatially resolved analysis of metabolites and proteins has become feasible within recent years by the development of mass spectrometry imaging (MSI) strategies. Development and application of these techniques has been pioneered in medicinal and pharmacological research. MS imaging allowed the detection of novel clinical markers for better diagnosis of cancer tissues and to follow the spatial-temporal patterns of drug molecules used for pharmacological studies (Rauser et al., 2010; Schwamborn et al., 2010).

The application of MSI strategies has now also been introduced in plant research. As higher plant organs are composed of a multitude of tissues, information on the spatial distribution of proteins and metabolites will be essential to generate improved models of metabolism and to assign biochemical functions of specific tissues. Sample preparation is a major bottleneck for successful MSI of plant tissues. The majority of the MSI studies on plants published to date have addressed the spatial distribution of certain classes of metabolites or of peptides or small proteins. Application of MSI of large proteins still provides considerable difficulties in practice. In the paper current strategies to address these limitations in MSI of plant proteins will be discussed. The particular advantages of MSI set-ups such as matrix-assisted laser desorption ionization (MALDI) MS or desorption electrospray ionization (DESI) MS will be highlighted. Approaches complementary to the MSI strategies will be briefly mentioned, in particular the use of laser microdissection of defined areas of tissue sections. Finally, data evaluation and integration into modeling approaches will be addressed.

MSI SAMPLE PREPARATION

Any sample preparation technique for MSI analyses aims on keeping the lateral resolution and the nature of the target molecules. According to the applied MSI technique it varies from just mounting a sample for surface analysis to delicate sectioning and matrix application procedures. Some recent publications provide detailed protocols on plant sample preparation for small molecule MSI (Peukert et al., 2012), as well as for protein MSI (Grassl et al., 2011). Strategies for optimized sectioning have been proposed such as varying conditions for sample freezing (e.g., dry ice for water rich samples), section mounting (e.g., embedding in water or gelatin for tiny or flat samples), variation of section thickness (10- to 35-μm), or section drying. Washing steps applied on the sectioned samples will impact on the classes of molecules retained on the tissue surface. Small molecules can be removed to improve imaging of peptides and proteins (Kaspar et al., 2011). Also, the choice of matrix and application strategy strongly influences the sort of molecular species which are ionized and the lateral resolution of the MS images. An increasing number of matrices for various applications have been recently explored and the interested reader is kindly referred to relevant publications (Svatos, 2010; Greer et al., 2011; Kaspar et al., 2011). For reproducible matrix application the most widely used deposition techniques are spraying with a simple airbrush and the use of a dedicated instrument to obtain vibrational vaporization. In our hands vibrational vaporization using a commercial device (ImagePrep, Bruker Daltonics, Germany) is most suitable to adjust optimal spraying and drying times, such as needed for multiple matrix layers and tryptic digestion on tissue sections.

MSI TECHNIQUES

All MSI techniques represent surface analysis techniques which are based on desorption and ionization of molecules followed by their subsequent MS data recording (Chaurand, 2012). The most common technique applied for MSI of metabolites and peptides is MALDI MSI, involving the application of a suitable matrix substance on the surface (Caprioli et al., 1997). Other common ionization processes are DESI utilizing a solvent stream (Takáts et al., 2004), secondary ion mass spectrometry (SIMS) making use of an ion beam (Vickerman, 2011), and laser ablation electrospray ionization (LAESI) (Nemes and Vertes, 2012). As DESI and LAESI techniques operate exclusively under atmospheric pressure (AP), sample preparation and associated issues are minimized. Most SIMS and MALDI sources operate in a vacuum chamber, which leads to the loss of volatile compounds and requires careful sample preparation (Chughtai and Heeren, 2010; Kaspar et al., 2011). Cryosectioning is a common procedure to prepare tissue sections for subsequent MALDI MSI (please refer to section MSI Sample Preparation). The spatial resolution currently achieved differs between the various ionization techniques. The highest spatial resolution ($<1\,\mu$) of current instruments is achieved for SIMS. Due to high fragmentation and low ionization efficiency, the size of biological molecules detected by SIMS analysis is limited. Instead, MALDI MSI has been favored with a current limit for spatial resolution of about 10 μm (Lee et al., 2012). Spatial resolution in MALDI MSI is influenced by the laser spot size, but is also strongly dependent on matrix application. Formation of large matrix crystals will negatively impact the spatial resolution to be achieved for the sample (Svatos, 2010; Peukert et al., 2012). DESI and LAESI techniques can be applied when the surface chemistry of the sample itself is of interest. Due to the low input for sample preparation, these approaches are suitable of screening larger sample sets (Svatos, 2010).

Various commercial mass analyzers are available for MSI providing sufficient: (i) mass resolution, (ii) spatial resolution, and (iii) MS scan speed. However, selection of one or another technique remains a compromise as none of the available mass analyzers meets perfectly all criteria, e.g., high resolution mass spectrometer typically have slower scan speed (Lee et al., 2012). The recent implementation of tandem mass spectrometry has encouraged MSI applications in plant research by enabling the identification of metabolites and, via on tissue digestion, N-terminal peptide derivatization and CID tandem MS, by facilitating the identification of polypeptides (Horn et al., 2011; Lunsford et al., 2011; Muller et al., 2011).

MSI OF SMALL MOLECULES

Imaging of small molecules including different classes of primary and secondary metabolites are the most frequent applications to date within plant MSI (Burrell et al., 2007; Kaspar et al., 2011; Lee et al., 2012; Peukert et al., 2012). Studies with a focus on method development and technology application on metabolites that are readily accessible for analysis are still highest abundant among available publications. However, an increasing number of experimental applications became available recently and an overview is presented in **Table 1**. Differential distribution pattern have been evaluated for a number of molecular species, namely, lipids, amino acids, and sugars, as well as high abundant secondary metabolites, such as polyphenols, anthocyanins, alkaloids, and glucosinolates from a variety of plant species. In the following, we will describe a number of selected examples in more detail.

Most analyses still rely on MALDI MSI techniques with variant matrices applied. For example MALDI MSI was applied to visualize the lipid species in cotton seed tissues (Horn et al., 2012). The comprehensive MSI study demonstrated distinct spatial patterns for molecular species of triacylglycerols and phosphatidylcholine. The MSI data set contained also information on a wide range of other lipid molecules, such as phosphatidylethanolamines, phosphatidic acids, sterols, and gossypol, supporting the wide applicability of the imaging approach (Horn et al., 2012). Also, non-uniform distribution of glucosinolates within *Arabidopsis* leaves was revealed by MALDI MSI (Shroff et al., 2008). Repeated spraying of the matrix 9-aminoacridine allowed the extraction of glucosinolates from the tissue beneath the surface. The major glucosinolates of the leaves were more abundant in tissues of the midvein and in the periphery when compared to the inner lamina. This pattern seemed to determine the feeding preference of insect larvae for the inner lamina (Shroff et al., 2008).

The application of colloidal graphite (GALDI) enabled the MSI of various small molecules in *Arabidopsis* surfaces and tissue sections (Cha et al., 2008). When analyzing leaf surfaces, very long chain fatty acids (C26, C28, and C30) were observed. Mass signals diagnostic for flavonoids, which localized within the cells, were only observed at positions were the leaf samples were damaged. When the surface epicuticular waxes were removed by dipping the leaves briefly into chloroform, the signals for fatty acids were strongly reduced. Instead, ions corresponding to kaempferol derivatives became apparent. MSI of flower leaves and stem sections also demonstrated heterogeneous distribution of flavanoids in this organs (Cha et al., 2008).

Matrix free UV-laser desorption/ionization (LDI) MSI at the single cell level provided information on the spatial distribution of UV-absorbing secondary metabolites for *Arabidopsis thaliana* (kaempferol derivatives) and *Hypericum* species (phloroglucinols and naphthodianthrones) (Hoelscher et al., 2009). The authors thoroughly confirmed and complemented the MSI data by analysis of isolated glands obtained through microdissection (Hoelscher et al., 2009).

Barley leaf tissue was subjected to MSI using direct and indirect DESI (Li et al., 2011) revealing a homogeneous distribution of hydroxynitrile glucosides. For direct DESI, the epidermis was stripped off and its back was analyzed. Indirect DESI was performed on imprints from intact leaves and of peeled epidermal strips using porous teflon. The indirect approach allowed relative quantification of these compounds in three divergent barley cultivars, namely Mentor, Golden Promise, and Emir (Li et al., 2011).

Only recently approaches have been published that utilize a combination of MALDI MS for imaging and high resolution MS for identification. MALDI MSI in combination with linear ion trap MS was required to study the distribution of the complex polymers cellulose and hemicelluloses in poplar tissue (Lunsford et al.,

Table 1 | Published literature describing the application of MS imaging for the analysis of small molecules from plant material.

Plant material	Applied technique	Applied matrix	Molecular species	Reference
Arabidopsis thaliana				
Leaf	MALDI MSI	9-Aminoacridine	Glucosinolates	Shroff et al. (2008)
Leaf	MALDI MSI	Lithium DHB[1]	Neutral lipids	Vrkoslav et al. (2010)
Leaf	LDI MSI	None	Flavonoids	Hoelscher et al. (2009)
Flower parts	GALDI MSI	Colloidal silver/colloidal graphite	Epicuticular lipids	Jun et al. (2010)
Flower petal	GALDI MSI	Colloidal graphite	Flavonoids	Perdian et al. (2010)
Root	GALDI MSI	Colloidal silver/colloidal graphite	Alkyl esters of coumarate, caffeate and ferulate, sterols	Jun et al. (2010)
Stem/flower/leaf	GALDI MSI	Colloidal graphite	Flavonoids, cuticular waxes	Cha et al. (2008)
Leaf/flower	GALDI MSI	Colloidal silver	Epicuticular waxes	Cha et al. (2009)
Triticum aestivum				
Stem	MALDI MSI	α-CHCA[2]	Oligosaccharides	Robinson et al. (2007)
Grain	MALDI MSI	α-CHCA, 9-aminoacridine	Amino acids, sugars, sugar phosphates	Burrell et al. (2007)
Hordeum vulgare				
Grains	MALDI MSI	DHB	Lipids	Peukert et al. (2012)
Leaf	DESI MSI	None	Hydroxynitrile glucosides	Li et al. (2011)
Oryza sativa				
Grain	MALDI MSI	DHB	Lipids, γ-oryzanol, phytic acid	Zaima et al. (2010)
Hypericum perforatum				
Leaf/flower parts	LDI MSI	None	Naphthodianthrones	Hoelscher et al. (2009)
Leaf/petal	DESI MSI	None	Secondary metabolites	Thunig et al. (2011)
Solanum melongena				
Fruit	MALDI MSI	DHB	γ-Aminobutyric acid, amino acids, sugars	Goto-Inoue et al. (2010)
Nicotiana tabacum				
Leaf	IR AP[3] MALDI MSI	None	Phenolics, alkaloids, oxylipins, sugars, among others	Ibanez et al. (2010)
Stem	MALDI MSI	DHB	Lipids	Peukert et al. (2012)
Solanum tuberosum				
Tubers	MALDI MSI	DHB	Glycoalkaloids	Ha et al. (2012)
Helianthus annuus				
Stem	MALDI MSI	α-CHCA	Nicosulfuron (pesticide)	Anderson et al. (2010)
Glycine max				
Leaf/stem	MALDI MSI	α-CHCA/SA	Azoxystrobin (fungizide)/mesotrione (herbicide)	Mullen et al. (2005)
Phoenix sp. (date palm tree)				
Leaf	MALDI MSI	Lithium DHB	Neutral lipids	Vrkoslav et al. (2010)
strawberry/banana/grapes				
Fruits	IR AP MALDI MSI	None	Sugar monomers and oligomers, citric acid	Li et al. (2007)
Gossypium hirsutum				
Seed/embryo	MALDI MSI	DHB	Lipids	Horn et al. (2012)

(Continued)

Table 1 | Continued

Plant material	Applied technique	Applied matrix	Molecular species	Reference
Populus sp.				
Stem	MALDI MSI	DHB	Oligosaccharides, polysaccharides	Lunsford et al. (2011)
***Vaccinium ashei*(blueberry)**				
Fruit	MALDI MSI	DHB	Anthocyanins	Yoshimura et al. (2012)
Malus sp. (Golden delicious)				
Fruit	MALDI MSI	α-CHCA	Flavonoids, dihydrochalcones	Franceschi et al. (2012)
Myristica malabrica(Lam)				
Fruit	DESI MSI	None	Alkaloids	Ifa et al. (2011)
Stevia rebaudiana				
Leaf	DESI MSI	None	Diterpenes	Gray et al. (2009)

[1] *2,5-Dihydroxybenzoic acid.*

[2] *α-Cyano-4-hydroxycinammic acid.*

[3] *Infrared atmospheric pressure.*

2011). MS spectra alone provided an even distribution of cellulose and hemicelluloses ions; however, when plotting characteristic fragment ions obtained by MS/MS, quite contrasting images were obtained. The authors concluded that tandem MS is necessary to separate isobaric species in order to accurately annotate wood tissue MS images. They also observed reduced background in the MS/MS experiments, which improved the signal-to-background ratio in the image analysis (Lunsford et al., 2011).

MSI ANALYSIS OF PEPTIDE AND PROTEIN PATTERNS

Mass spectrometry-based imaging of proteins is of particular interest for biomedical research. However, the mass range for sensitive detection of proteins in tissue sections is limited. Identification of proteins in imaging experiments is still a challenging task. On tissue digestion using proteolytic enzymes is possible; conversely this procedure increases the complexity of the MS spectra considerably. This fact necessitates high accurate mass measurements (Schober et al., 2011). Another challenge is to keep spatial resolution during tryptic digestion of the proteins on the tissue surface. A protocol has been developed which allowed to achieve a spatial resolution of 50 μm (Schober et al., 2012).

So far, few reports describe the MSI analysis of peptides from plant tissues. *In situ* MALDI MS analysis determined the structure of a modified 12-amino acid peptide (MCLV3), which was derived from a conserved motif in the CLV3 sequence in *Arabidopsis* callus (Kondo et al., 2006). The spatial distribution of cyclotide peptides was analyzed by MALDI MSI in *Petunia* leaves (Poth et al., 2012). Cyclotides represent a family of plant peptides and are characterized by a structural feature called the cycline cystine knot. Several reports suggest a role of cyclotides in plant defense. The non-uniform distribution of cyclotides in *Petunia* leaves would be consistent with such a role (Poth et al., 2012). Additionally, discriminative peptides in barley grain sections were highlighted as examples in a recent review (Kaspar et al., 2011).

Most protein MSI studies to date are from the field of medicine (Caprioli et al., 1997; Yanagisawa et al., 2003; Schwartz et al., 2005; Goodwin et al., 2008). Spatial distribution of proteins can be used as markers for diagnosis of diseases and to better differentiate between diseased and healthy tissues. Unknown proteins can serve as valuable markers and assist diagnosis and disease treatment. Their identification will be necessary to build a biochemical network representing the molecular events underlying the development of the disease (Mascini and Heeren, 2012). Targeted MSI for proteins was recently performed by means of the combination of immunohistochemistry with MALDI MSI using single chain fragment variable recombinant antibodies (Thiery et al., 2012).

The first report on protein MSI for plants described the detection and identification of the allergenic lipid transfer protein Pru p3 in the peel of the peach fruit by means of electrospray MS identification and MALDI MSI (Cavatorta et al., 2009). Method development for MALDI MSI of proteins was recently published, encouraging the wider application of protein MSI in plant tissues (Grassl et al., 2011). Main constraints for MSI of intact proteins present the rather low protein abundance, the high water content, and the rigid cell walls and abundant air spaces in many plant tissues, resulting mainly in challenging sample preparation. As for the presented soybean sample, the authors propose to prepare and freeze the sample using dry ice immediately after collection to preserve morphology and minimize protein degradation through proteolysis and to avoid breakage and cracking as observed during shock freezing by liquid nitrogen. Optimal section thickness for soybean seedlings has been found to be 10- to 15-μm from frozen sections. However embedding in gelatin showed an improvement in localization, lateral resolution, and reproducibility, with some

Table 2 | Schematic representation of the current status of MSI in plant science.

Plant Material	Sample Preparation	MSI Approach	Image Analysis	Identification Strategies
• Leaf • Stem • Root • Flower • Fruit • Parts of organs (hairs, pollen, etc.) • Single cells	*Tissue analysis:* • Sectioning	• MALDI MSI • LDI MSI	• Data preprocessing • Ion intensity maps • Multivariate analysis	*Direct approaches:* • MALDI MSI + linear ion trap MS (only shown for small molecules)
	Direct and indirect surface analysis: • Tissue imprinting • Tissue mounting	• DESI MSI • IR AP MALDI MSI • GALDI MSI • DESI MSI • IR AP MALDI MSI		*Indirect approaches:* • On tissue digestion + MS/MS of peptides • Laser microdissection +targeted analyses (e.g. LC-MS, GC-MS)

*For molecular species targeted by the different approaches please refer also to **Table 1**.*

loss in signal-to-noise. Soaking tissues in sucrose, and thus filling of air spaces between cells, dramatically improves cryosectioning and the lateral resolution during imaging. A wash with ice-cold 2-propanol enabled the fixation of proteins, and removed lipids and salts substantially. Complete drying of the tissue sections was also shown to be important in order to preserve protein localization. Best reproducibility in ion intensity as well as spatial resolution was observed when using sinapinic acid (SA) as a matrix. Matrix application was performed by means of vibrational spray (Image Prep, Bruker Daltonics, Germany) with an optimized protocol for spraying and drying cycles. For MALDI MSI measurement the authors suggested to adjust a resolution of 30–100 μm for the laser raster points. In addition, the authors comprehensively reviewed approaches enabling the identification of MSI protein targets either "on tissue" or by extraction procedures aiming to conserve the spatial localization (Poth et al., 2012). However, none of these identification approaches has been successfully applied to plants so far.

EVALUATION OF MSI DATA SETS

Analysis of multiple tissue sections at high spatial resolution necessarily generates large data sets providing challenges for the subsequent data mining. A number of imaging software packages are available, both open source (e.g., BioMap, see http://www.maldi-msi.org/) as well as commercial solutions (Kaspar et al., 2011). Still the visualization as well as the statistical treatment of large data sets requests further developments. Identification of unknown compounds requires high resolution mass spectrometry. Frequently, additional efforts are necessary to annotate compounds of interest, such as targeted analysis of micro-dissected materials or other complementary approaches. Software capable of handling three-dimensional datasets will be another essential tool for visualization.

FURTHER DEVELOPMENT OF MS IMAGING

Spatial resolution of current instrumentation for LDI/MALDI MSI is restricted to 10–20 μm. MSI at cellular and in particular sub-cellular resolutions requires improvements in the techniques. Recently, the Caprioli group has developed transmission geometry MALDI MS allowing submicron spatial resolution (Zavalin et al., 2012). As a feature, the transmission geometry vacuum ion source enabled to irradiate the back of the sample with the laser beam. The development of this laser optics together with an adjusted sample preparation protocol allowed sufficient sensitivity of the instrument also at submicron spatial resolution (Zavalin et al., 2012). Further implementation of MSI will benefit from such specific developments, but also from the overall advances still seen in bio-analytical mass spectrometry instrumentation.

CONCLUSION

Mass spectrometry imaging has recently been introduced into plant sciences mostly focused on the spatial distribution of low molecular weight compounds, including primary and secondary plant metabolites as well as cyclic peptides (**Table 2**). These studies will encourage extension of the approach toward other plants systems and applications. Sample preparation, selection of matrix substances and application of the matrix are critical to obtain images of sufficient quality using MALDI MSI. DESI MSI together with a number of other aforementioned imaging approaches provides strategies with complementary applications. Most promising are future developments in tandem MS technologies, such as combining MALDI MSI with high resolution MS for identification, and thus enabling the correlation of molecular distribution pattern to particular molecular networks and tissue function, and the quantitation of differential distributions. The studies already published will guide the further implementation of tandem MSI techniques for plant samples and extend the range of possible applications.

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Organ-specific proteome analysis for identification of abiotic stress response mechanism in crop

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Abiotic stresses, such as flooding, drought, salinity, and high/low temperatures, are the major constraints that global crop production faces at present. Plants respond to a stress by modulating abundance of candidate proteins, either by up-regulating expression or by the synthesizing novel proteins primarily associated with plant defense system. The cellular mechanisms of stress sensing and signal transduction into cellular organelles have been reported. Nevertheless, the responses of plant cells to abiotic stresses differ in each organ. As the correlation between the expression of mRNAs and the abundance of their corresponding proteins is difficult to assess in specific organs, proteomics techniques provide one of the best options for the functional analysis of translated regions of the genome. The present review summarizes the organ-specific proteome analyses for better understanding of the response mechanisms of crops to abiotic stresses, including flooding, drought, and salinity. The differential organ-specific responses against each of these stresses are discussed in detail to provide new insights into plant stress response mechanisms at protein level.

Keywords: crop, proteomics, organ-specific, abiotic stress, flooding, drought, salinity

INTRODUCTION

Abiotic stress is a key limiting factor that impairs growth and yield of agricultural crops around the world (Hossain et al., 2012). Stressful environment may lead to delay in seed germination, reduced seedling growth, and finally decreased crop yield. Under abiotic stress, various compounds are either synthesized in the roots such as hormones and amino acids and/or taken up such as water and mineral nutrients by the roots and must be transported to the shoot to enable normal leaf functioning (Ghanem et al., 2011). Proteins associated with the primary function of an organ, are uniquely expressed in that specific organ/tissue (Watson et al., 2003). This organ-specific expression of proteins is thus essential for plant growth and development. Organ-specific proteomics analyses help us in better understanding the response mechanisms of plants toward abiotic stresses.

In higher plants, leaves represent highly specialized organ that is primarily engaged in photosynthesis. It has been shown in several reports that rates and activities of photosynthesis are highly dependent on the development and age of the leaf, and this is also correlated with the accumulation of proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and other photosynthesis-related proteins (Maayan et al., 2008; Urban et al., 2008). Leaves also play major role in transporting essential elements and water from the roots to aerial parts. Plant roots can sense gravity, water, nutrients, and other signals in the soil. Moreover, they have the ability to secrete numerous compounds that protect the root apex and regulate root growth (Feldman, 1984; Aiken and Smucker, 1996). A better understanding of the mechanisms determining root length and branching is expected to improve crop production/yields (Lynch, 2007). Abiotic stress initially affects the underground or overground part of a plant and is sensed by the roots or leaves that finally trigger cellular signal transduction pathways leading to molecular and metabolic changes. Hence, for better understanding of how plants respond and adapt to abiotic stresses, it is important to focus on the root and leaf system.

Analysis of organ-specific protein abundance provides rich information about the response mechanisms of plants to abiotic stress. However, total protein extraction from leaves is difficult due to the presence of abundant proteins, which also interfere with the expression analysis of low-abundance proteins by physically or chemically masking their abundance. In contrast to leaves, the concentration of proteins in roots is relatively low, also making protein identification difficult. The direct homogenization of plant organs with a solubilization buffer has not yielded high-quality proteome maps for leaves or roots, suggesting that the direct extraction method is not suitable for plant proteins. Hence, more research needs to be focused on the improvement of protein extraction methods for obtaining high resolution organ based proteome map.

Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; 2-DE, two-dimensional polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; ROS, reactive oxygen species; MS, mass spectrometry, MRM, multiple reaction monitoring.

PROTEIN EXTRACTION FROM ORGANS

To magnify the expression of low-abundance proteins, the elimination of high-abundance proteins from samples is compulsory. Using a polyethylene glycol (PEG)-fractionation

method, RuBisCO, which is an abundant protein in the leaf, was eliminated from other leaf proteins during extraction (Ahsan et al., 2007a). In this method, proteins were first extracted from tomato leaves using Mg/Nonidet P-40 buffer consisting of 0.5 M Tris-HCl, 2% Nonidet P-40, 20 mM $MgCl_2$, 2% 2-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride, and 1% polyvinyl polypyrrolidone, and were then fractionated with 15% PEG (Ahsan et al., 2007a). Hashimoto and Komatsu (2007) reported the preparation of an anti-RuBisCO LSU antibody-affinity column with protein A-Sepharose as a resin. The leaf protein extract was incubated with purified IgG, and was further incubated with rehydrated protein A-Sepharose resin. The resin was then added to a Spin X cup (Pierce, Rockford, IL, USA), centrifuged, and washed several times with the wash solution. Because the collected flowthrough from the wash solution contained released IgG molecules, these solutions were applied to the Protein A Agarose kit column (KPL, Gaithersburg, MD, USA) to trap the released IgG, thereby removing the extra IgG molecules (Hashimoto and Komatsu, 2007).

For root proteomics studies, a number of enrichment methods have been used due to presence of high levels of low-abundance proteins in roots. The most widely used protein extraction method is trichloroacetic acid (TCA)/acetone precipitation. Using this method, Nanjo et al. (2012) extracted proteins from roots with a solution of 8 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine. Ahsan and Komatsu (2009) reported that treatment of root with Mg/Nonidet P-40 buffer followed by extraction with alkaline phenol and methanol/ammonium acetate produced high-quality proteome maps consisting of numerous well-separated spots with high intensity, on two-dimensional polyacrylamide gel electrophoresis (2-DE) gels.

Various protein extraction and solubilization methods have been evaluated for obtaining high-quality, reproducible proteome reference maps of various plant organs. For example, when seed proteins of soybean were extracted by TCA/acetone precipitation, substantial horizontal streaking was observed in 2-DE gels (Mooney et al., 2004). This result suggests that the TCA/acetone precipitation method does not completely remove non-protein contaminants from plant organs (Komatsu and Ahsan, 2009). Although both TCA/acetone- and phenol-based methods are reliable and efficient methods for extracting proteins from various plant organs (Rose et al., 2004; Espagne et al., 2007), experimental evidence for various types of soybean organs has demonstrated that the phenol-based method gives reproducible, high-quality proteome maps compared to other available methods (Ahsan and Komatsu, 2009). In recent time, gel-free proteomics techniques are often being used for proteome analysis; however, the purification of protein extracts using methods such as TCA/acetone precipitation is needed.

LEAF PROTEOMICS ANALYSIS UNDER ABIOTIC STRESS

LEAF PROTEOMIC ANALYSIS UNDER FLOODING STRESS

The negative impacts of flooding include inhibition of leaf growth, reduction of biomass production, and ultimately, reduced seed yield. Flooding can also result in reduced stomatal conductance and decreased chlorophyll a and b content in leaves (Gomes and Kozlowski, 1980). The decrease in chlorophyll content was more significant in older leaves, suggesting that chlorophyll degradation proceeds more rapidly in leaves that are closer to flooded roots. The reduction of plant biomass in response to flooding may be directly related to stomatal limitations on net photosynthesis that result in reduced carbon assimilation (Mielke et al., 2003). Restriction of photosynthetic activity may also be influenced by changes in the components of the biochemical reactions, such as RuBisCO and other photosynthesis-related proteins (Maayan et al., 2008). Proteomics analyses of leaf tissue have revealed that the majority of the identified proteins are involved in energy production and primary/secondary metabolism (Donnelly et al., 2005).

Ahsan et al. (2007a) examined protein changes in tomato leaves exposed to waterlogging stress and found that the expression of proteins associated with stress/defense mechanisms and energy/metabolism were increased, while photosynthesis- and protein biosynthesis-related proteins were decreased. Among the identified photosynthesis-related proteins, the expression of RuBisCO was decreased in the total soluble proteins in response to waterlogging stress. RuBisCO has a dual function: it acts as a carboxylase mediating photosynthetic CO_2 assimilation and as an oxygenase catalyzing the first step of the photorespiratory pathway. Waterlogging stress-induced decrease in abundance of RuBisCO activase protein has also been reported in tomato leaves (Ahsan et al., 2007a) that maintains RuBisCO in an active, functional state. In addition, the authors reported that submergence stress induced the formation of reactive oxygen species (ROS) that ultimately leads to degradation of the subunits of RuBisCO and RuBisCO activase (Ahsan et al., 2007a). Inhibition of protein biosynthesis and activation of proteases in the tomato leaves were found to be the major causes of injuries due to waterlogging. In addition, increased expression of heat shock proteins and other stress-related proteins was also observed, indicating that activation of the defense system promoted survival under submerged conditions (Ahsan et al., 2007a).

Khatoon et al. (2012) investigated the organ-specific response mechanism in 1-week-old soybean seedlings under flooding stress by analyzing protein profiles in the roots, hypocotyls, and leaves using a gel-based proteomics technique. Among a total of 577 protein spots identified in leaves, 24 and 26 spots were increased and decreased, respectively, in response to flooding stress. Compared to untreated seedlings, 16 protein spots exhibited more than a two-fold change, with 6 spots increasing and 10 spots decreasing. In leaves, more metabolism-related, cytoplasmic, and chloroplastic proteins were decreased than were increased, while all of the disease/defense-related proteins were decreased. As compared to the roots and hypocotyls, fewer energy-related proteins were observed in leaves. Among the reduced metabolism-related proteins, isoflavone reductase, which plays an essential role against oxidative injuries, was identified. Isoflavone reductase is involved in the biosynthesis of alkaloids that play important roles in defense against various stresses (Kajikawa et al., 2009). The reduced levels of isoflavone reductase not only in leaves, but also in roots, is one of the factors involved in the decreased efficiency of the antioxidant system in soybean seedlings exposed to flooding stress. The decrease of isoflavone reductase and several other disease/defense-related proteins in the

roots and leaves of flooded seedlings compared to non-stressed seedlings indicates that the defense response is highly suppressed in soybean seedlings under flooding stress.

LEAF PROTEOMIC ANALYSIS UNDER DROUGHT STRESS

Stomatal closure in response to drought stress primarily results in a reduced rate of photosynthesis. Environmental conditions that increase the transpiration rate also tend to increase the pH of leaf sap that in turn, promotes abscisic acid accumulation and lead to reduced stomatal conductance (Davies et al., 2002; Wilkinson and Davies, 2002). Very severe drought conditions limit photosynthesis due to a decline in RuBisCO activity (Bota et al., 2004). The activity of the photosynthetic electron transport chain is finely tuned to the availability of CO_2, and photosystem II activities often declines in parallel under drought conditions (Loreto et al., 1995). Proteomic analyses of leaves from rice (Salekdeh et al., 2002a,b; Ali and Komatsu, 2006; Ke et al., 2009), sugar beet (Hajheidari et al., 2005), wild watermelon (Yoshimura et al., 2008), tall wheatgrass (Gazanchian et al., 2007), *Quercus ilex* (Echevarría-Zomeno et al., 2009), *Populus euramericana* (Bonhomme et al., 2009), wheat (Caruso et al., 2009), sunflower (Castillejo et al., 2008), and soybean (Mohammadi et al., 2012a,b) have identified numerous drought-responsive proteins, which were chiefly involved in redox regulation, oxidative stress response, signal transduction, protein folding, secondary metabolism, and photosynthesis. Vincent et al. (2005) used a proteomics approach to understand the impact of drought stress on the lignification of maize leaves, and revealed that proteins involved in lignification and flavonoid synthesis have an important contribution to the plant response to water deficit.

LEAF PROTEOMIC ANALYSIS UNDER SALINITY STRESS

Soil salinity is an ever-present threat to crop yield, especially in arid and semi-arid zones. Salinity, chiefly due to the presence of NaCl, is considered the single most widespread soil toxicity problem of global crop production (Hossain et al., 2006). On exposure to salt stress, expression of most of the photosynthesis-related proteins in leaves were found to be decreased, suggesting that NaCl adversely affects photosynthesis and energy production, and consequently reduces plant growth. Proteomic analyses of leaf tissue from rice (Salekdeh et al., 2002a,b; Abbasi and Komatsu, 2004), citrus (Tanou et al., 2009), glasswort (Wang et al., 2009), *Suaeda aegyptiaca* (Askari et al., 2006), and soybean (Sobhanian et al., 2010) have identified a variety of salt-responsive proteins. Among these proteins, abundance of calreticulin, a calcium-binding chaperone protein that plays a pivotal role in regulating calcium homeostasis and protein folding in the endoplasmic reticulum (Menegazzi et al., 1993; Wang et al., 2004), was decreased in rice leaves under osmotic stress (Zang and Komatsu, 2007). This finding indicates that calcium is an important secondary messenger in rice seedlings exposed to salt stress. The principal role of RuBisCO activase is to release inhibitory sugar phosphates, such as ribulose-1,5-biphosphate, from the active sites of RuBisCO to allow its activation by CO_2 through carbamylation (Jordan and Chollet, 1983). Additionally, RuBisCO activase functions as a chaperone during stress (Rokka et al., 2001). In rice leaves, the levels of RuBisCO activase were decreased upon exposure to NaCl. This might be the prime reason of declined photosynthetic activity under NaCl stress (Parker et al., 2006). Dysfunction of protein is a common consequence of abiotic stress. Molecular chaperones/heat-shock proteins are responsible for proper protein folding, assembly and translocation (Wang et al., 2004). Although most newly synthesized proteins can fold in the absence of chaperones, a minority strictly requires assistance of these specialized proteins (Horvath et al., 2008). The 20-kDa chaperonin functions as a co-chaperone, along with cpn60, and in certain cases is essential for the discharge of biologically active proteins from cpn60 (Bertsch et al., 1992). Sobhanian et al. (2010) reported abundance of a 20-kDa chaperonin was increased in the leaves of soybean seedlings exposed to salt stress, suggesting the protective roles of chaperones in preventing the misfolding of proteins under salt stress. In addition, a decrease of the 50S ribosomal subunit, which catalyzes the peptidyl-transfer reaction of messenger RNA-directed protein biosynthesis (Kotusov et al., 1976), indicates that NaCl has an inhibitory effect on soybean protein biosynthesis and presumably leads to the observed reduction in plant growth under high salinity.

ROOT PROTEOMIC ANALYSIS UNDER ABIOTIC STRESS

ROOT PROTEOMIC ANALYSIS UNDER FLOODING STRESS

Proteomic analyses of root including hypocotyl of 2-day-old soybeans exposed to flooding stress revealed that proteins related to glycolysis including UDP-glucose pyrophosphorylase and fructose-bisphosphate aldolase, disease/defense-related proteins such as ROS scavengers, chaperones, hemoglobin, and/or acid phosphatase, were highly affected (Hashiguchi et al., 2009; Komatsu et al., 2009; Nanjo et al., 2010). The flooding response mechanism in soybean during late growth stages was investigated in 3-week-old soybean seedlings subjected to flooding for 3 and 7 days (Alam et al., 2010). The study revealed that levels of enzymes of the glycolysis and fermentation pathways were mostly affected. This finding suggests that soybean seedlings respond similarly to flooding stress during early and late growth stages.

Kong et al. (2010) investigated the response mechanism of 2-day-old wheat roots exposed to flooding stress. The observed decrease in proteins involved in glycolytic pathway supports the notion that reduced carbohydrate metabolism and energy consumption are the primary responses that plants use to cope with flooding stress. In addition, proteins related to disease/defense were increased in abundance, while cell wall structure/modification-related proteins, including methionine synthase, were decreased, suggesting that cell growth was restricted to save energy in the unfavorable environment imposed by flooding. Haque et al. (2011) examined the response mechanism of wheat to flooding stress at different root depths, and found that proteins showing increased expression were related to energy and redox status, defense responses, and cell wall turnover. Based on the proteomic data, it was suggested that these proteins were possibly involved in alternative respiration and cell degeneration for promoting metabolic adjustment in response to the hypoxia stress-induced by flooding. Ahsan et al. (2007b) reported that tomato plants could cope with the severe conditions under submergence. In particular, proteins related

to secondary metabolite biosynthesis, programmed cell death, and diseases/defense were increased in flooded tomato roots (Ahsan et al., 2007b). Metabolic adjustment for the management of energy consumption during cellular processes was the key adaptive response in tomato roots exposed to submergence stress, as evidenced by the increase of alcohol dehydrogenase and enolase, and decreased level of pyruvate dehydrogenase (Ahsan et al., 2007b). Proteomics based screenings of proteins conferring tolerance against submergence has provided novel information for future development of genetically engineered flood-tolerant crops.

ROOT PROTEOMIC ANALYSIS UNDER DROUGHT STRESS

Root development is strongly influenced by adverse growing conditions; however, root growth is typically less affected by drought stress than shoot growth (Franco et al., 2011). Thus, a decrease in the shoot: root ratio is commonly observed under drought stress, and results from either increased root growth or from a relatively larger decrease in shoot growth compared to root growth. In addition, roots typically contain a greater percentage of fine roots, which are capable of penetrating smaller soil pores and presumably optimize the exploratory capabilities of the root system as a whole, and also may have an important role in survival against drought. Proteomic analysis of the root in rice (Mirzaei et al., 2012), soybean (Toorchi et al., 2009; Alam et al., 2010; Mohammadi et al., 2012a), and *Brassica napus* (Damerval et al., 1988; Mohammadi et al., 2012b) under drought stress has identified a wide range of proteins, that lead to a better understanding of the mechanism of drought stress tolerance in plants.

Mirzaei et al. (2012) investigated how rice root systems in heterogeneous soils adapt to drought by comparing root tissues under four conditions: (1) fully watered; (2) fully droughted, and split-root systems where (3) one-half was watered and (4) the other half was droughted. Label-free proteomic analysis of these four kinds of roots resulted in the identification of 1487 non-redundant proteins, with nearly 900 proteins present in each treatment. Drought caused marked changes in expression, most notably in partially droughted roots, in which the levels of 38% of proteins were altered compared to adjacent, watered roots. In response to drought, pathogenesis- related proteins were generally increased. In contrast, heat-shock proteins were not detected in roots of fully watered plants. Proteins involved in transport and oxidation-reduction reactions were also highly dependent upon drought signals, with transport-related proteins largely absent in roots receiving a drought signal while oxidation-reduction proteins were typically increased during drought. The comparison showed that nine tubulins were strongly reduced in droughted roots, while the levels of six chitinases were increased, even when the signal arrived remotely from adjacent, droughted roots. This label-free proteomic analysis of water stress in split-root systems of rice has provided novel molecular insights into the heterogeneous translation patterns that occur in wet and dry soil zones.

ROOT PROTEOMIC ANALYSIS UNDER SALINITY STRESS

Root represents the vital plant organ that first encounters salt stress. Some salt stress-responsive genes and proteins are strongly induced in roots than in other organs (Yan et al., 2005). Proteomic analyses of the root in soybean (Sobhanian et al., 2010), rice (Liu et al., 2012), wheat (Guo et al., 2012), maize (Zörb et al., 2010), barley (Sugimoto and Takeda, 2009), and potato (Aghaei et al., 2008) under salt stress have been reported. These studies have revealed that a number of salt stress-responsive genes are more strongly induced in roots than in other organs.

Sobhanian et al. (2010) examined the proteome change of soybean roots under high salinity and observed that the expression of several metabolism-related proteins were mainly decreased under salt stress. Among the decreased proteins, a dienelactone hydrolase, which hydrolyzes the conversion of dienelactone to maleylacetate, which are both intermediates for the aerobic degradation of haloaromatic compounds (Schlomann et al., 1990; Blasco et al., 1995), was identified, suggesting that these secondary metabolites are not effectively degraded under salt stress. Sugimoto and Takeda (2009) performed proteomic analysis of specific proteins in root of salt-tolerant barley. In salt-tolerant barley, six proteins were identified as stress/defense-related proteins that do not scavenge ROS directly, and they had been reported in other plants, indicating that in course of time a common salt tolerance mechanism might have developed in plants.

ROOT TIP PROTEOMIC ANALYSIS UNDER ABIOTIC STRESS

Root is the main organ for water and nutrient absorption, and for anchorage of the plant. Longitudinally, root can be divided into three different regions: those of cell division, elongation, and maturation (Howell, 1998). The region of cell division, the root tip, contains the root apical meristem, where cells divide, but do not elongate immediately. Cell elongation and maturation in the root are thought to be controlled by the extensibility of the cell wall and the turgor pressure inside the cell (Cosgrove, 1996). The tip portion of the primary roots is important for seedling establishment (Drew et al., 1994). Proteomics analysis of the root apex revealed that the most abundant proteins are involved in stress response, glycolysis, redox homeostasis, and protein processing (Mathesius et al., 2011). All of the identified proteins of root apex were also reported to be present in differentiated root zones, but with different abundance. Flooding induced cell death in the 5-mm-long root tip region and suppressed root elongation were reported in flooded soybean seedlings (Nanjo et al., 2012).

Suppression of root elongation and induction of root tip cell death under flooding stress have also been reported in maize and pea (Subbaiah and Sachs, 2003; Gladish et al., 2006). Proteome analysis of the root tip under flooding stress was performed in soybean seedlings using mass spectrometry (MS)-based quantitative proteomics and phosphoproteomics approaches (Nanjo et al., 2012). A number of the differentially changed proteins, including sucrose-binding protein, phosphatidylinositol-4-phosphate 5-kinases, actins, and alpha-tubulins, were expressed specifically in the root tip region under flooding stress. The increased expression of sucrose-binding proteins in flooded soybean root tips implies that sucrose accumulated in roots, a finding that was reported previously in soybean roots, including the hypocotyl (Nanjo et al., 2010). A relationship between proteolysis and flooding stress was demonstrated in the root tip using a proteomics approach (Yanagawa and Komatsu, 2012). The study

findings indicated that proteolytic processes involving ubiquitin/proteasomes occur in roots under flooding stress, leading to degradation of root tip cells and death of the root cap cells. Furthermore, it was demonstrated that flooding, not the hypoxic conditions, were responsible for the root tip degradation resulting from ubiquitin/proteasome-mediated proteolysis, as these injuries were independent of the oxygen concentration (Yanagawa and Komatsu, 2012).

When considering the changes in the protein abundance induced by flooding in the root tip compared to those in the root and hypocotyl, it can be inferred that the abundance of various proteins was specific to the root tip. In particular, the abundance of actins and alpha-tubulins, which are involved in cytoskeleton remodeling, was observed in only the root tip. The decrease of these proteins in the root tip suggested that they play a role in the suppression of root elongation during flooding (Nanjo et al., 2012). Further, it is clear that plant adaptation to flooding stress has involved the change of proteins in specific regions of the root, leading to the inhibition or acceleration of various biological processes, which collectively enables plants to cope with flooding stress. Proteins overrepresented in the root apex were primarily involved in the pathways for protein synthesis and processing, cell redox homeostasis, and flavonoid biosynthesis, while underrepresented proteins were those involved in glycolysis, tricarboxylic acid metabolism, and stress response. These results highlight the importance of stress and defense responses, redox control, and flavonoid metabolism in the root apex (Mathesius et al., 2011).

DIFFERENTIAL CHANGES IN COMMON PROTEINS AMONG ROOTS, HYPOCOTYLS AND LEAVES UNDER ABIOTIC STRESS

Proteins that are commonly expressed in different plant organs under stress provide valuable information for designing genetically engineered stress tolerant crop plants. Comparison of organ-specific proteome changes on exposure to flooding stress has been well-documented in soybean. Khatoon et al. (2012) analyzed the flood induced changes in protein species using an organ-specific proteomics approach. The 2-DE separation of extracted proteins from roots, hypocotyls and leaves of 2-day-old soybeans subjected to flooding, revealed significant changes in 51, 66, and 51 protein species, respectively. In all three organs, proteins primarily involved in energy, metabolism, and disease defense showed altered expression. Among the differentially changed proteins, abundance of isoflavone reductase was commonly decreased in roots, hypocotyls and leaves under flooding stress (**Figure 1**). Interestingly, expression of phosphoglycerate kinase was decreased both in roots and hypocotyls while increased in leaves. Out of the four common protein species between root and hypocotyl, phosphoglycerate kinase and methionine synthase were decreased; while fructose-bisphosphate aldolase was increased under submergence. Elongation factor 1-delta protein expression was decreased and increased in root and hypocotyl, respectively. Interestingly, in both roots and leaves, Kunitz trypsin protease inhibitor was increased under flooding. In addition, Enolase was decreased in both hypocotyls and leaves in response to flooding. The remaining proteins exhibited organ-specific changes in abundance under flooding, which suggest that response mechanism of each organ in soybean seedlings is differently affected by flooding stress (**Figure 1**). At transcript level isoflavone reductase gene was up-regulated in leaves; while at protein level it showed decreased abundance. The difference in abundance of leaf isoflavone reductase between transcript and protein levels indicates that flooding stress affects the process of protein turnover in flooded seedlings. Their findings suggest that flooding stress lead to imbalance in expression of isoflavone reductase, involved in the biosynthesis of plant defense metabolites- lignins and isoflavonoids, along with other metabolism and disease/defense related proteins, might impair the growth of various organs in soybean seedlings under flooding stress.

Changes in protein levels in drought and PEG induced osmotic stressed soybean seedlings were analyzed using similar organ-specific proteomics approach (Mohammadi et al., 2012a,b). Among the 3 organs, root was found to be the most drought-responsive organ, with 32, 13, and 12 proteins with changed abundance in response to drought stress, PEG treatment, and both, respectively. In leaves abundances of metabolism-related proteins were increased while energy production- and protein synthesis related proteins were decreased. Findings revealed that a total of 3 proteins were commonly expressed in leaves, hypocotyls and roots of drought stressed soybean seedlings. Heat shock protein 70 and actin isoform B were upregulated and methionine synthase was downregulated under drought irrespective of the organ type (**Figure 1**). The observed downregulation of mRNA and declined protein levels of methionine synthase in leaves, hypocotyls, and roots of drought-stressed plants, but not in response to heat and salinity treatments indicate that methionine synthase is a drought responsive protein. All these findings suggest that the low abundance of methionine synthase might be responsible for poor growth of soybean seedlings under drought stress.

Sobhanian et al. (2010) similarly exploited proteomic techniques to unravel the effects of salt stress on organ-specific protein abundance in soybean. Exposure to 40 mM NaCl stress resulted alterations in 19, 22, and 14 protein abundances in the leaves, hypocotyls and roots, respectively. In all three organs, metabolism, and energy related proteins were largely down-regulated in response to salinity (**Figure 1**). Notably, glyceraldehyde-3-phosphate dehydrogenase and fructokinase were down-regulated in leaves/hypocotyls and hypocotyls/roots, respectively. In contrast, stem 31 kDa glycoprotein precursor was up-regulated in all three organs with NaCl treatment.

Taken together, all these organ-specific proteomics findings indicate that metabolism- and energy-related proteins in addition to defense play pivotal roles in each organ for adaptation to adverse environmental conditions.

CHALLENGES AND FUTURE PROSPECTS

The present review outlines the impact of abiotic stresses on organ-specific proteome constituents. As the correlation between the expression of mRNAs and the abundance of their corresponding proteins is difficult to assess in specific organs, proteomics techniques provide one of the best options for the functional analysis of translated regions of the genome. Analysis

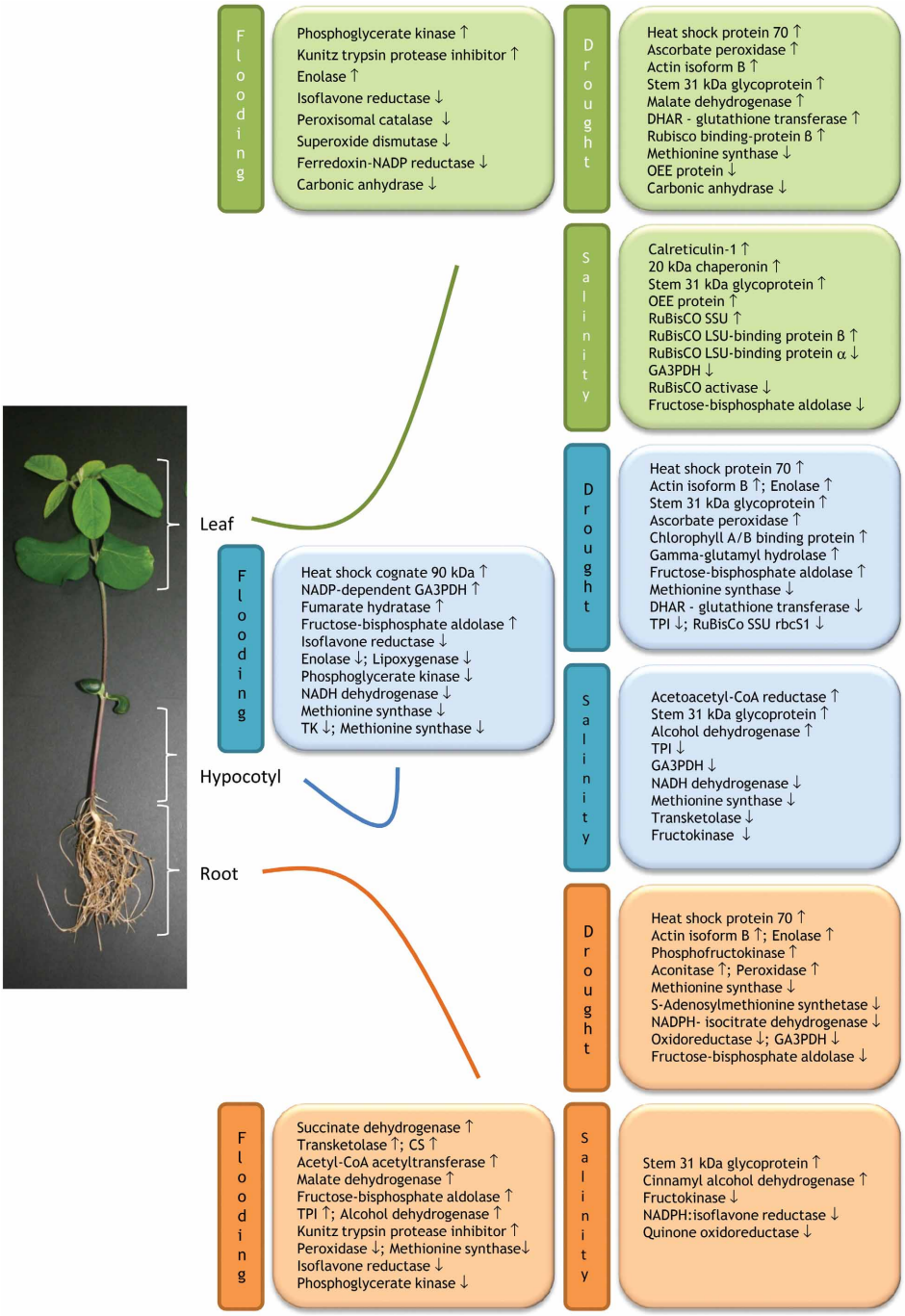

FIGURE 1 | Schematic illustration of organ-specific expression of proteins in response to drought, flooding, and salinity stresses. The scheme is based on the published organ-specific proteomic works on soybean (*Glycine max* L.) under the mentioned abiotic stresses. Up and down arrows indicate stress-induced increase and decrease protein abundance, respectively. Abbreviations: CS, Chalcone synthase; DHAR, dehydroascorbate reductase; GA3PDH, glyceraldehyde-3-phosphate dehydrogenase; LSU, large subunit; OEE, oxygen-evolving enhancer; SSU, small subunit; TK, Transketolase; TPI, Triosephosphate isomerase.

of changes in organ-specific protein abundance strengthens our knowledge for better understanding the response mechanisms of plants to abiotic stress. Most of the investigations done so far primarily highlighted the over all organ response against a given stress. However, to pin point the stress response mechanisms of plants, changes in proteome compositions in the specific target areas within the whole organ, especially in case of roots need to be explored further.

Poor proteome coverage is often considered as the main bottleneck of organ proteomic study. While studying leaf proteome,

changes in stress-responsive proteins are difficult to detect on 2-DE gel due to the presence of an abundant protein RuBisCO LSU, which accounts for about 50% of the total proteins. Fractionation of crude protein extract is the most promising technique to reach better proteome coverage. Use of anti-RuBisCO LSU antibody-affinity column to trap RuBisCO LSU protein has been an effective method to make the protein extract free of RuBisCO LSU (Hashimoto and Komatsu, 2007). This technique allowed the authors to identify four additional cold-responsive proteins in leaves of rice seedlings. More initiatives in the advancement of fractionation technique need to be taken to cope with the limited proteome resolution.

Study of changes in organ-specific proteome composition by conventional 2-DE approach coupled with MS provides a broad idea about plant stress response mechanism. However, to dissect the stress responsive biochemical pathways, targeted approaches need to be explored further. Identification of low abundance of signaling proteins and transcription factors, their protein complexes is often a challenge for classical 2-DE based organ proteomic technique. Smaczniak et al. (2012) recently reported a sensitive, quantitative proteomics based procedure to determine the composition of plant protein complexes. Fluorophore-tagged protein immunoprecipitation and label-free MS-based quantification techniques facilitate identification of low abundance signaling and regulatory protein complexes from native plant tissues. In addition, advanced technique like laser-capture microdissection (Dembinsky et al., 2007) for tissue proteomics could be exploited further to facilitate identification of tissue- and cell-specific proteins involved in plant responses to abiotic stress.

In systems biology, there has always been a growing demand of an accurate quantification of target sets of proteins across multiple samples (Picotti et al., 2009). Selected reaction monitoring (SRM), the most emerging targeted and highly sensitive MS technique, has great potential for the reliable identification and accurate quantitation of very low-abundance proteins in complex biological mixtures and characterization of modified peptides (Calvo et al., 2011; Picotti and Aebersold, 2012). Statistical and computational tools are essential for designing and analysis of SRM experiments, particularly in analyses of large sample (Chang et al., 2012). Picotti et al. (2009) demonstrated the potential of SRM-based proteomics technique by the consistent and fast measurement of a network of proteins spanning the entire abundance range over a growth time course of *S. cerevisiae* transiting through a series of metabolic phases. This targeted proteomics approach facilitated the detection and quantification of low abundance proteins expressed to a concentration below 50 copies/cell in total *S. cerevisiae* digests. Very recently, Picotti et al. (2013) exploited a strategy based on high-throughput peptide synthesis and MS to generate an almost complete reference map (97% of the genome-predicted proteins) of the *S. cerevisiae* proteome. The low-copy-number proteins (<103 copies/cell) play key biological roles in regulation of cellular processes or signal transduction. To elucidate a complex protein network pathway, each individual minor constituent protein needs to be identified with accuracy. The outstanding multiplexing abilities, reproducibility, sensitivity and selectivity make SRM an invaluable tool in targeted proteomics for determining very subtle expression changes, thus facilitates protein network modeling.

Improvements in multiple reaction monitoring (MRM) MS technique provide new insights into plant stress signaling pathways. Rainteau et al. (2012) used MS in the MRM mode to analyze the fatty acid composition of the major glycerophospholipids in Arabidopsis suspension cells. Findings reveal that phospholipases D action in response to salicylic acid is not due to the production of a stress-specific molecular species, but that the level of phospholipases D products *per se* is important. The over-representation of two species in phospholipases D products compared to putative substrates was linked to a regulatory role of the heterogeneous distribution of glycerophospholipids in membrane sub-domains (Rainteau et al., 2012). It is suggested that MRM-MS strategy constitutes a reliable method for determining the extract composition of glycerophospholipids in plants, essential to unravel the cell stress signaling pathway.

All these new findings indicate that, in coming days, amalgamation of diverse MS techniques coupled with bioinformatics technology with improved sample preparation and fractionation strategies would provide us more precise and comprehensive picture about plants stress response mechanism. In conclusion, more attention need to be paid in future organ-specific proteomic research to unravel those target proteins that are commonly expressed in most organs under wide range of abiotic stresses. The findings could shed some light on the cross talk between different abiotic stress signal pathways. All these valuable information would further enable us to design genetically engineered stress tolerant crop plants.

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Subcellular protein overexpression to develop abiotic stress tolerant plants

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Environmental stresses are major factors limiting growth and development of crops. Plants respond to the stresses through a wide range of reactions from morphological changes to alterations in the patterns of protein expression. Understanding the mechanisms involved in the stress response is the first step to develop abiotic stress tolerant crops. Proteomics is a powerful tool in evaluating regulated proteins in the cell under stress and it is an efficient technique in studying stress tolerant plants. Because of the nature of abiotic stress, intracellular compartments play a main role in the stress response. Subcellular proteins such as ion and water transporters, reactive oxygen species (ROS) scavengers, and the proteins related to signaling and transcriptional regulation are frequently reported as being involved in stress tolerance. Overexpression of stress-responsive protein through generation of transgenic plants is one the main practical approaches in production of tolerant plants. In this article, recent studies on transgenic plants overexpressing subcellular proteins are reviewed and the role of organelles and over-expressed proteins is classified.

Keywords: subcellular protein, transgenic, overexpression, abiotic stress

INTRODUCTION

Adverse environmental conditions threaten normal growth and development of plants. Abiotic stresses mainly including temperature extremes, drought, and salinity detrimentally affect plant growth and crop yield. It was reported that for most crops, abiotic stresses are leading to a reduction in the average yield by more than 50% (Bray et al., 2000). One-third of the world's population resides in water-stressed regions, and because of climate changes, water stress could become more frequent and severe in the future (Manavalan et al., 2009). Adverse abiotic stresses tend to occur together and they are almost never present individually in nature. On the other hand, plant cells likely follow similar mechanisms to cope with abiotic stresses. For instance, cold, drought, and salinity are well-known to the generation of reactive oxygen species (ROS) causing oxidative stress (Dat et al., 2000). Therefore, ROS scavenging genes are candidate in the generation of plants with tolerance to the stress.

At the cellular level, plants adopt a wide range of responses to cope with abiotic stress. The mechanism associated with sensing stress, transduction of stress signals into the cell is well-known, and it represents the initial reaction of plant cells to stress (Desikan et al., 2004). Stress signals are first encountered by the outer parts of the cell under a highly organized process for sensing environmental changes (Heino and Palva, 2003). ROS, which are formed by partial reduction of molecular oxygen during abiotic stress, are recognized as a signal to activate the defense response (Vranová et al., 2002). Transduction of the signal into the cell through cascades alters gene and protein expression, leading to physiological responses. Communication through intracellular compartments plays an important role in this process.

The major subcellular organelles and compartments in plant cells are nucleus, mitochondria, chloroplasts, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, and plasma membrane. The intracellular organelles and their interactions during stressful conditions represent the primary defense response. In fact, communication between organelles and cytosolic and luminal proteins renders the protein composition of organelles dynamic (Agrawal et al., 2011). Most receptor proteins are located in the plasma membrane, and thus the plasma membrane is directly involved in stress sensing (Komatsu, 2008). Intracellular organelles with high capacity for production of ROS, such as mitochondria and chloroplasts are the primary sites for the production of this signaling molecule. ROS can also increase the effects of cellular damage (Dat et al., 2000; Van Breusegem et al., 2001). According to the role of cellular organelles and compartments, there are several key subcellular proteins involved in stress tolerance in plant cells.

Proteome analysis of cellular organelles under abiotic stress indicated that the accumulation of responsible proteins highly improves plant tolerance to the stress. Therefore, overexpression of related genes through the production of transgenic plants is a powerful technique to enhance stress tolerance in plants. Generation of tolerant transgenic plants against abiotic stresses has extensively been discussed in reviews (Wang et al., 2003; Bartels and Sunkar, 2005). However, classification of subcellular-localized proteins and their related genes involved in abiotic stress

response is scant. This review highlights the studies in recent decade on abiotic stress tolerant transgenic plants overexpressing subcellular proteins.

ABIOTIC STRESS-RELATED PROTEINS IN PLANT CELL

When the plant is exposed to abiotic stress, the proper function of the cell will be highly affected. Cell survival under stress condition directly depends on how the cell can adapt itself to the environment. Cellular defense mechanisms against abiotic stress are mainly controlled by the expression of responsible genes and proteins. Generally, many plants are susceptible to unfavorable environmental conditions. Therefore, overexpression of protective genes might help to reduce the deleterious effects of the stress. Generation of transgenic plants overexpressing the stress-responsive gene and protein will confer stress resistance leading to enhanced plant growth and productivity (Allen et al., 1997). Three major groups of genes are reported to be involved in the stress response. The first group are those that are involved in signaling cascades and in transcriptional regulation. The second group are those having a role in the protection of membranes and proteins and the third group are those involved in water and ion uptake and transport (Wang et al., 2003).

In order to know about major subcellular proteins conferring stress tolerance, it is important to understand the roles of organelles themselves in the cell. Organelles such as chloroplast and mitochondria are mainly responsible for metabolic processes including photosynthesis, photorespiration, oxidative phosphorylation, and the tricarboxylic acid cycle (Taylor et al., 2009). The plasma membrane is mainly involved in stress signal perception, transducing into the cell, and ion and water transport. The tonoplast is involved in ion balance and adjustment of water content. The nucleus has a variety of functions including transcriptional regulation, signaling, and gene regulation (Hossain et al., 2012). Despite the particular roles of organelles, their coordinated functions and interactions are important in the stress response. Hossain et al. (2012) classified the defense-related abiotic stress-responsive proteins into six major groups according to their functions. The protein groups are osmoprotectant regulators, ROS scavengers, ion transporters, water channels, molecular chaperones, and proteolysis-related proteins. Most of the protein groups are functionally attributed to organelles or compartments indicating the importance of subcellular proteins in response to stress conditions.

Apart from changes in the expression of organelle proteins, post-translational modifications (PTMs) of proteins are also known as a defense mechanism against abiotic stress. Redox proteomics is an increasingly emerging branch of proteomics aimed at identifying PTMs, particularly under stressful conditions. Recent proteome studies analyzed a number of redox-targets at organelles, which offered a view of the proteome modifications that are regulated by abiotic stress. For example, several proteins are identified as a target for S-nitrosylation in peroxisomes (Ortega-Galisteo et al., 2012) and mitochondria (Camejo et al., 2012) of pea plants under abiotic stress conditions. The modification of organelle proteins could regulate H_2O_2 level or modulate the respiratory and photorespiratory pathways in the plant.

OVEREXPRESSING OF ORGANELLE PROTEINS IN TRANSGENIC PLANT IMPROVES TOLERANCE TO ABIOTIC STRESS

Transgenic plants overexpressing the genes encoded subcellular-localized proteins are classified (**Table 1**). Stress-responsive genes or proteins were arranged according to the localization of the overexpressed protein in the cell. Nucleus, chloroplast, plasma membrane, ER, mitochondria, and vacuole are the organelles and compartments in which the presence of overexpressed proteins was confirmed (**Table 1**). Among the organelles, near half (42%) of the overexpressed proteins was localized in the nucleus (**Figure 1A**). This means that the genes encode nuclear proteins were highly considered in the generation of transgenic plants. According to the role of nucleus protein under abiotic stress, it can be postulated that the molecular mechanism of stress tolerance in most of the studied transgenic plants is based on transcriptional regulation, signaling, and gene regulation. Other overexpressed proteins were mainly localized in chloroplast (23%), plasma membrane (13%), and ER (10%) (**Figure 1A**). This classification performs a preliminary ranking of the importance of organelles in response to abiotic stress, regardless of the function of overexpressed protein.

Classification of the transgenic plant species indicated that out of 30 overexpressed proteins, *Arabidopsis*, rice, and tobacco were applied in 16, 6, and 5 studies, respectively (**Table 1**). Interestingly, while overexpressed proteins of *Arabidopsis* and tobacco were localized in diverse organelles, the overexpressed proteins of rice were only localized in the nucleus and chloroplast. Bartels and Sunkar (2005) classified stress-responsive genes contributing to drought or salt tolerance in transgenic plants. They reported a similar order for a number of successful transgenic *Arabidopsis*, tobacco, and rice. This result indicates that despite great advances in the generation of transgenic plants, most of the transgenic plants are among the model plants.

Overexpression of a specific protein in transgenic plant confers a level of tolerance to the plant. A diverse range of stresses that are mainly related to unfavorable environmental conditions is classified as abiotic stress. Overexpressed gene or protein in most of the transgenic plants is contributing to salt (36%) and drought (29%) tolerance which means 65% of all studies were only focused on salt and drought tolerance. Tolerance to other abiotic stresses such as cold (13%), oxidative (7%), and heat (5%) were relatively less reported (**Figure 1B**). Manavalan et al. (2009) described the importance of water stress at the present and in the future. Therefore, researchers are working more on the generation of salt and drought stress tolerant transgenic plants.

EFFECTS OF PROTEIN OVEREXPRESSION ON CELLULAR MECHANISMS OF PLANT

A wide range of responses has been reported in transgenic plants subjected to abiotic stress. Alterations in morphology, physiology, and cellular mechanisms of transgenic plants are summarized (**Table 1**). The articles listed in the table, mainly did not comprehensively characterize cellular responses to the stresses. Therefore, it is difficult to perform functional classification of the overexpressed genes or proteins. However, growth enhancement in transgenic plants compared to wild type can be

Table 1 | Overexpression of subcellular proteins in transgenic plants to enhance tolerance to abiotic stress (Since 2000).

No.	Localization	Protein (gene)	Abiotic stress	Effects on plant/cellular mechanism	Plant species	References
1	Nucleus	Cold shock protein (*CSP*)	Cold, heat, drought	Improve in plant growth, yield	*Arabidopsis thaliana, Oryza sativa*	Castiglioni et al., 2008
		R1R2R3 MYB TF (*OsMYB3R-2*)	Freezing, drought, salt	Tolerance to NaCl and ABA during seed germination	*Arabidopsis thaliana*	Dai et al., 2007
		Late embryogenesis abundant protein (*OsLEA3-2*)	Salt, drought	Improve in germination speed, plant growth	*Oryza sativa*	Duan and Cai, 2012
		R2R3 MYB transcription factor (*AtMYB44*)	Drought, salt, cold	Water loss reduction	*Arabidopsis thaliana*	Jung et al., 2008
		ethylene-responsive factor like protein 1 (*CaERFLP1*)	Salt	Enhance tolerance	*Nicotiana tabacum*	Lee et al., 2004
		TaMYB2A (*TaMYB2A*)	Drought, salt, freezing	Enhance cell membrane stability, photosynthetic rate, reduction of osmotic potential	*Arabidopsis thaliana*	Mao et al., 2011
		OsNAC6 (*OsNAC6*)	Drought, salt	Transcriptional activator	*Oryza sativa*	Nakashima et al., 2007
		WRKY-type transcription factor (*TaWRKY2, TaWRKY19*)	Drought, salt	Regulation of STZ or DREB2A-mediated pathways	*Arabidopsis thaliana*	Niu et al., 2012
		Ethylene-responsive element binding protein (*AtEBP*)	Oxidative, heat	Suppression of Bax-induced cell death	*Nicotiana tabacum*	Ogawa et al., 2005
		Trihelix transcription factor (*GmGT-2A, GmGT-2B*)	Salt, drought, freezing	Improve in seedling morphogenesis	*Glycin max*	Xie et al., 2009
		R2R3-MYB transcription factor (*OsMYB2*)	Drought, salt, cold	Accumulation of more soluble sugars and proline	*Oryza sativa*	Yang et al., 2012
		Basic leucine zipper transcription factor (*AtbZIP24*)	Salt	Osmotic and ionic balance	*Arabidopsis thaliana*	Yang et al., 2009
2	Chloroplast	β-carotene hydroxylase (*DSM2*)	Drought	Control of the xanthophyll cycle and ABA synthesis	*Oryza sativa*	Du et al., 2010
		Glycine betaine (*codA*)	Salt, cold, oxidative	Enhance in plant growth, repair of photo-damaged PSII	*Oryza sativa, Lycopersicon esculentum*	Su et al., 2006; Park et al., 2007
		Homogentisate phytyltransferase (*HPT1*)	Light	Tocopherol regulation	*Arabidopsis thaliana*	Collakova and DellaPenna, 2003
		Betaine aldehyde dehydrogenase (*BADH*)	Salt, oxidative, cold	Higher photosynthetic activity, reduction of ROS	*Ipomoea batatas*	Fan et al., 2012

(Continued)

Table 1 | Continued

No.	Localization	Protein (gene)	Abiotic stress	Effects on plant/cellular mechanism	Plant species	References
		Plastidal protein synthesis elongation factor (*Zmeftu1*)	Heat	Reduced thermal aggregation of leaf proteins, reduced heat injury to thylakoids, enhanced rate of CO_2 fixation	*Triticum aestivum*	Fu et al., 2008
		Chloroplast small heat shock protein (*Cpshsp*)	Cold	Less electrolyte leakage and less destruction of chlorophyll, higher photosynthetic rate	*Lycopersicon esculentum*	Wang et al., 2005
		Aldehyde dehydrogenase (*ALDH3I1*)	Salt, drought	ROS scavenger, reduced the level of lipid peroxidation	*Arabidopsis thaliana*	Kotchoni et al., 2006
3	Plasma membrane	Na^+/H^+ antiporter (*SOS1*)	Salt	Less Na^+ accumulation, improve yield	*Arabidopsis thaliana, Zea maiz*	Shi et al., 2002; Chen et al., 2007
		Aquaporin (*PIP1b*)	Drought (-)*	Higher plant growth, transpiration rate, stomatal density and photosynthetic efficiency, plant vigore	*Nicotiana tabacum*	Aharon et al., 2003
		Nodulin 26-like intrinsic protein (*TaNIP*)	Salt	Higher K^+, Ca^{2+} and proline contents and lower Na^+ level	*Arabidopsis thaliana*	Gao et al., 2010
		Sucrose non-fermenting 1-related protein kinase 2 (*TaSnRK2.8*) **	Drought, salt, cold	Higher relative water content, cell membrane stability, PSII activity	*Arabidopsis thaliana*	Zhang et al., 2010
4	Endoplasmic reticulum	E3 ubiquitin ligase (*Rma1H1*)	Drought	Inhibiting aquaporin trafficking	*Arabidopsis thaliana*	Lee et al., 2009
		Annexin (*AnnAt1 and AnnAt4*)	Drought, salt	Improve in plant growth	*Arabidopsis thaliana*	Huh et al., 2010
		BiP (soy *BiP*)	Oxidative	Tolerance to the glycosylation inhibitor tunicamycin, tolerance to water deficit	*Nicotiana tabacum*	Alvim et al., 2001
5	Mitochondria	Glycine-rich RNA-binding protein 2 (*GRP2*)	Salt, cold, osmotic stress	Higher seed germination, seedling growth and freezing tolerance	*Arabidopsis thaliana*	Kim et al., 2007
		Uncoupling protein (*AtUCP1*)	Drought, salt	Higher germination, photosynthesis, respiration, leaf water content	*Nicotiana tabacum*	Begcy et al., 2011
6	Vacuole	Na^+/H^+ antiporter (*AtNHX1, TNHX1*)	Salt, drought	Higher photosynthesis, nitrogen assimilation	*Arabidopsis thaliana, Gossypium hirsutum*	Brini et al., 2007; He et al., 2005
		ERD six-like1 (*ESL1*)	Drought, salt, ABA	Efflux of hexoses from the vacuole	*Arabidopsis thaliana*	Yamada et al., 2010

*Minus indicates the negative effects of overexpressed protein under stress condition.

**This protein co-localized in nucleus, cytoplasm, and plasma membrane.

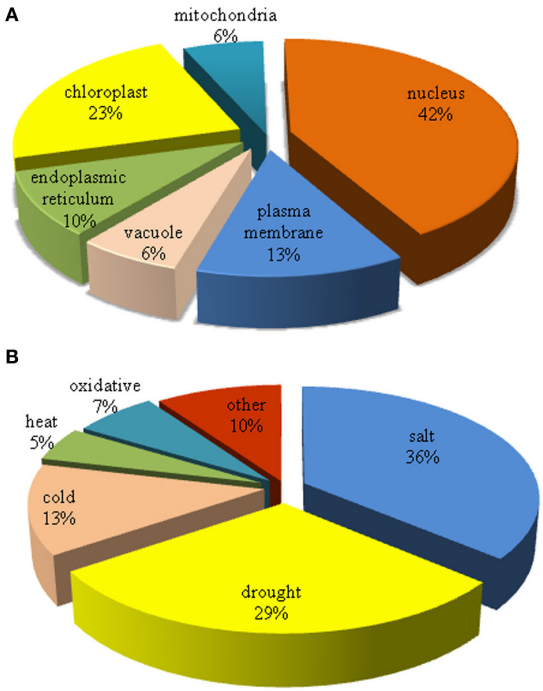

FIGURE 1 | Subcellular localization of overexpressed protein and type of abiotic stress is classified. (A) Percentage of organelles in which overexpressed proteins were localized. **(B)** Distribution of abiotic stresses in which transgenic plants were tolerant.

considered as a general morphological response. Improvement of the photosynthetic activity is another mechanism involved in stress tolerance. Proteins including MYB transcription factor (TaMYB2A) (Mao et al., 2011), glycine betaine (Park et al., 2007), betaine aldehyde dehydrogenase (Fan et al., 2012), chloroplast small heat shock protein (Wang et al., 2005), Sucrose non-fermenting 1-related protein kinase 2 (Zhang et al., 2010), and even Na^+/H^+ antiporter (Brini et al., 2007) enhance photosynthetic activity. The proteins related to photosynthesis were not only localized in the chloroplast but also they were overexpressed in the other organelles.

Overexpression of some proteins in transgenic plant might have negative effects on plant tolerance to abiotic stress. Creissen et al. (1999) reported that in transgenic tobacco overexpressing chloroplast-targeted γ-glutamylcysteine synthetase (γ-*ECS*), foliar levels of glutathione were significantly raised. This protein as a major antioxidant in most aerobic organisms supposed to protect the photosynthetic apparatus from oxidative damage. Paradoxically, however, increased glutathione biosynthetic capacity in the chloroplast resulted in greatly enhanced oxidative stress possibly by failure of the chloroplast homeostatic mechanism (Creissen et al., 1999). Transgenic tobacco overexpressing aquaporin (*PIP1 b*) is another example of the susceptibility of transgenic plant to abiotic stress. Aquaporin improves plant growth rate, transpiration rate, stomatal density, and photosynthetic efficiency under favorable growth conditions. However, overexpression of aquaporin reduced plant growth under drought stress because of rapid wilting (Aharon et al., 2003). Therefore, two main strategies in the generation of successful transgenic plants are; (1) consideration of the cellular and subcellular interactions of overexpressed protein; (2) selection of stress-specific target gene or protein according to the main role of the protein under stress condition.

CONCLUSIONS AND FUTURE PERSECTIVE

Abiotic stress is one of the main challenges in expansion of planting area worldwide. Application of omics technology such as proteomics, transcriptomics, and metabolomics together with bioinformatics are frequently reported in plant abiotic stress studies (Moumeni et al., 2011; Hakeem et al., 2012). Proteomics improved the efficiency of conventional breeding by identification of the stress-responsive proteins. Overexpression of specific stress-responsive protein through the generation of transgenic plant is an efficient technique which has been successfully applied in model plants. In this review, a survey of recent decade studies (from 2000) to generate transgenic plants overexpressing subcellular-localized proteins is presented. Classification of transgenic plants according to the organelle indicated that 42% of the overexpressed proteins were localized in the nucleus. Therefore, the nucleus functions such as transcriptional regulation, signaling, and gene regulation are more considered in the generation of the plants with tolerance to abiotic stress.

Furthermore, 23 out of the 30 stress-responsive proteins were overexpressed only in *Arabidopsis*, rice, or tobacco. This result suggests that despite great advances in the generation of transgenic plants, this technique is mainly applied in the model plants. Therefore, one of the challenges in the future would be adopting the technology of protein overexpression in economically relevant crops. However, our knowledge about cellular mechanisms against abiotic stress as a prerequisite needs to be improved. Rapidly developing omics technology has the potential to decipher the functions of cell and intracellular organelles against abiotic stress in future studies.

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Application of selected reaction monitoring mass spectrometry to field-grown crop plants to allow dissection of the molecular mechanisms of abiotic stress tolerance

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One major constraint upon the application of molecular crop breeding approaches is the small number of genes linked to agronomically desirable traits through defined biochemical mechanisms. Proteomic investigations of crop plants under abiotic stress treatments have identified many proteins that differ in control versus stress comparisons, however, this broad profiling of cell physiology is poorly suited to ranking the effects and identifying the specific proteins that are causative in agronomically relevant traits. Here we will reason that insights into a protein's function, its biochemical process and links to stress tolerance are more likely to arise through approaches that evaluate these differential abundances of proteins and include varietal comparisons, precise discrimination of protein isoforms, enrichment of functionally related proteins, and integration of proteomic datasets with physiological measurements of both lab and field-grown plants. We will briefly explain how applying the emerging proteomic technology of multiplexed selective reaction monitoring mass spectrometry with its accuracy and throughput can facilitate and enhance these approaches and provide a clear means to rank the growing cohort of stress responsive proteins. We will also highlight the benefit of integrating proteomic analyses with cultivar-specific genetic databases and physiological assessments of cultivar performance in relevant field environments for revealing deeper insights into molecular crop improvement.

Keywords: abiotic stress, molecular breeding, wheat, barley, rice, selected reaction monitoring, proteomics

CROP LOSSES DUE TO ABIOTIC STRESS AND THE PROMISE OF APPLYING MOLECULAR TECHNIQUES TO CROP IMPROVEMENT

Analyses by agricultural researchers and humanitarian organizations have continually found that abiotic stresses such as drought, extreme temperatures, and unfavorable soil conditions are responsible for significant decreases in crop yield and can have adverse economic and nutritional consequences for local populations (Boyer, 1982; Mittler, 2006; Witcombe et al., 2008). Given the vast increase in molecular understanding of plant biology over the last decade, as well as the successful application of molecular techniques in the field of biomedicine, it has been proposed that the approaches and techniques of molecular biology should be applied to crop improvement strategies in order to increase the abiotic stress tolerance of crops. It is hypothesized that these approaches would generate agronomically useful germplasm with greater speed and precision than classical breeding (Moose and Mumm, 2008; Tester and Langridge, 2010). The first step in any molecular breeding strategy involves defining a suite of candidate genes that possess molecular functions that will enhance the stress tolerance. To date, this has been the rationale of many plant proteomics studies carried out in a wide range of important crop plants. Here, we will critically assess the current state of crop proteomics research and its progress toward the aim of novel gene discovery for abiotic stress tolerance. We will propose a workflow that combines laboratory-based discovery proteomics followed by selected reaction monitoring (SRM) mass spectrometry (MS) of field-grown plants, with the aim of convincing readers that such an approach could contribute more relevant information to advance both gene discovery and gene evaluation for crop improvement programs (**Figure 1**).

A CRITIQUE OF PROTEOMIC INVESTIGATIONS INTO ABIOTIC STRESS TOLERANCE IN PLANTS

Cellular physiology is underpinned by the composition and function of the proteome, and the power of proteomics techniques lies in their ability to quantitatively analyze large numbers of proteins in parallel, providing rich sets of information with which to explain the molecular mechanisms that underpin cellular function. The plant proteomics literature is characterized by two major threads. The first is the descriptive cataloging of which proteins are expressed in which tissue (Baerenfaller et al., 2011; Lee et al., 2012), localized to which organelle (Heazlewood et al., 2007; Eubel et al., 2008; Taylor et al., 2011), or bound within which protein complex (Remmerie et al., 2011). The second thread is made up of comparative studies that identify differences in protein profiles between tolerant versus sensitive genotypes (Vincent et al., 2007; Jacoby et al., 2010; Pang et al., 2010), or between control plants and plants exposed to environmental stress conditions (Taylor et al., 2005; Kosova et al., 2011). The results from comparative studies are framed as gene discovery exercises, where proteins induced by stress or proteins of higher abundance in the tolerant genotype are

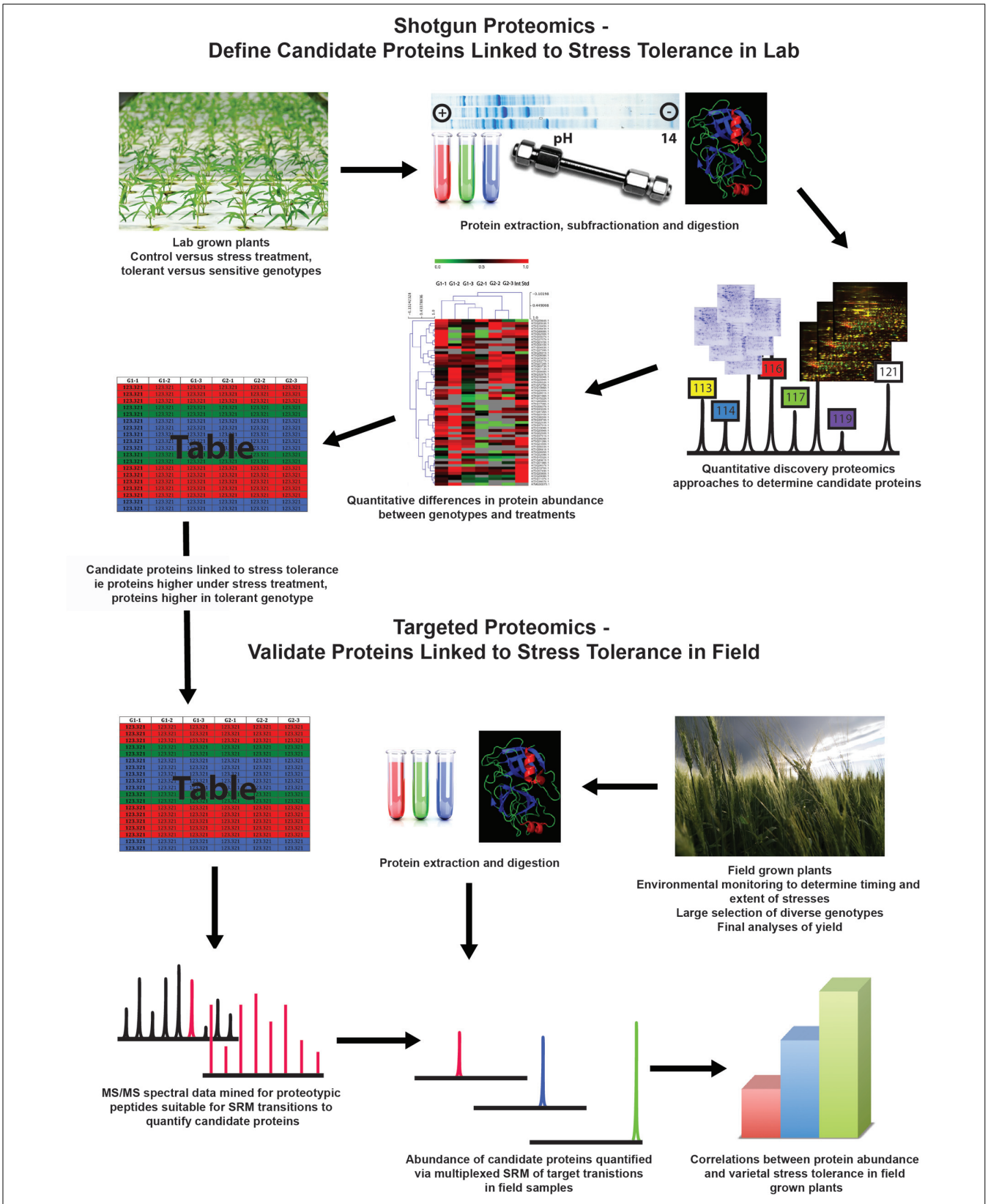

FIGURE 1 | Proposed workflow that combines laboratory-based discovery proteomics followed by selected reaction monitoring mass spectrometry of field-grown plants to gain more relevant information to advance both gene discovery and gene evaluation for crop improvement programs.

positioned as logical candidates for improving stress tolerance in crops, particularly when their molecular functions involve stress related processes such as redox defense or signal transduction. The information gathered by proteomic studies of these kinds has increased the scientific understanding of plant biology, with the results from descriptive proteomic studies being particularly valuable for accurately defining expression profiles across tissue and the subcellular localizations of proteins, while the results of the control versus stress experiments have defined many proteins which are induced in response to stress, significantly increasing our knowledge of how cellular physiology responds to environmental challenge.

However, the value of the above mentioned approaches as gene discovery and ranking strategies for future crop improvement is less clear. Many limitations of the approach and results derived from these experiments must be taken into account before committing to pursue any one protein in a molecular breeding program. First is the common observation that a small subset of the stress responsive proteins are repeatedly identified across a number of different stress experiments, the so called "déjà vu" phenomenon (Petrak et al., 2008). While these proteins undoubtedly play a role in stress response and will be consistently expressed under a wide range of stresses, and their repeated identification might be seen as evidence of their high value, it is likely they will be poor candidate genes for crop improvement, as it is likely that elite agronomic varieties will already increase expression of these proteins in response to stress due to their generalized stress responsiveness. Rather, it is the rarely observed changes in specific experiments that likely hold the key as useful gene traits. A second criticism is the common focus in proteomics papers on the proteins that increased in abundance, over and above those that decreased, and the ranking of fold changes as a proxy for importance. Given the diverse impact of changing the abundance of protein in the control of biochemical pathways, small changes can have large effects and large changes can have small effects, depending very much on the protein in question and its tissue distribution. A third criticism concerns the choice of genotypes and growth conditions used to generate tissue for many of these proteomics studies. To date, proteomics researchers have only sampled a very small portion of the wide diversity that exists between crop varieties, with many experiments repeatedly documenting the stress responses of reference genotypes (e.g., rice cv. Nipponbare, wheat cv. Chinese Spring, barley cv. Golden Promise), which are genetically well characterized, amenable to genetic transformation and have extensive genome sequence resources needed for proteomics identifications, but often show poor agronomic performance. The current focus upon this narrow range of germplasm may limit the range of physiological coping strategies that are being documented. Arguably, this may make the discovery of novel physiological coping strategies rare rather than common events in laboratory settings. More events might be found by analyzing the proteomic responses of diverse varieties adapted to a wider range of environments (Glaszmann et al., 2010). Fourthly, and somewhat similarly to the criticisms leveled at the diversity of genotypes used in studies, there is an obvious difference in timing, severity, and multiplicity of stress responses in plants growth in controlled environments chambers versus crops sown in the field. Most stress proteomic studies analyze the protein expression profiles of plants treated with one single stress under tightly controlled environment growth conditions, with only a smaller number of studies considering two or more stresses, but this contrasts with field-grown plants that will much more routinely experience numerous stresses of varying intensities, which often occur simultaneously (Mittler and Blumwald, 2010). The role of the field environment is particularly important in eliciting genotype × treatment (G × T) interactions, as it has been shown that certain genotypic differences in abiotic stress tolerance only manifest under field conditions (Richards et al., 2010; Tavakkoli et al., 2012). Of course, there are a number of sound reasons which have led proteomics researchers to generate tissue under controlled conditions, such as phenotypic reproducibility, the demand for sufficient quantities of homogeneous tissue, and proximity to laboratory facilities. Furthermore, it is very challenging to conduct reproducible stress treatments in the field to define clear protein targets against a changing background, due to spatial and temporal variations in climate and soils.

We propose that controlled environment experiments that attempt to mimic field conditions are best framed as a starting point to identify proteins of interest. The results of these studies then need to be added together and explored more widely by subsequent profiling experiments conducted under field conditions in the target environment, to determine which, if any, of the favorable molecular mechanisms uncovered in the laboratory hold true in the target environment and what are the relationships between them.

SELECTED REACTION MONITORING MASS SPECTROMETRY AS A MEANS TO PROFILE THE MOLECULAR MECHANISMS THAT UNDERPIN ABIOTIC STRESS TOLERANCE OF CROPS IN THE FIELD

The ongoing advancement of MS instrumentation and approaches such as sequential window acquisition of all theoretical fragment-ion spectra (SWATH; Gillet et al., 2012) enable current researchers to employ a wider range of methodological approaches. However, these new technologies should not be applied indiscriminately, as it is important to accurately match enhanced technical capabilities and knowledge of their limitations against a relevant biological question in order to generate novel and applicable scientific insights. Here we propose that the emerging plant proteomics application of peptide SRM is well suited to dissecting the molecular mechanisms that underpin phenotypic performance of crop plants in the field. To date this approach has been used to characterize sucrose synthase isoforms and N-metabolism enzymes in *Medicago* (Wienkoop et al., 2008), a basic amino acid carrier involved in arginine metabolism in rice (Taylor et al., 2010) and the plasma membrane transportome in *Arabidopsis* (Monneuse et al., 2011). A number of technical challenges are associated with the establishment of SRM approaches and these have been covered in detail in recent reviews (Picotti and Aebersold, 2012; Thompson et al., 2012). Some of these challenges are particularly pertinent in experiments planned in crop plants. For example it is imperative to determining the uniqueness of a peptide sequence, as valid results are not possible when a peptide used for quantitation is present in

more that one protein. In species with well-characterized genomes such as *Arabidopsis* or rice, this can be overcome with computational analysis (Rost et al., 2012). Generally the longer the peptide the more likely it is to be unique. However, in crop plants this remains a challenge, as without complete genome sequences confidence in a peptide's uniqueness would be limited. Further issues arise when attempting to distinguish between splice variants of a gene or enzyme isoforms. In these cases, it is likely that only very small regions of the proteins differ from one another and thus a limited number of peptides may be available for unique quantitation. Another level of complexity is introduced by the presence of missed cleavages during enzymatic digestion prior to triple-quadrupole (QqQ) MS and the presence of post-translational modification (PTM) sites within peptides selected for SRM. Overall much care must be taken when selecting peptides for quantitation by SRM, particularly in crop plants with incomplete genome sequences and limited knowledge on enzyme isoforms and potential PTMs.

We will highlight the features of SRM that are well matched to the types of biological questions currently being asked by proteomics researchers who seek to define links between protein abundance and abiotic stress tolerance. Firstly the use of a QqQ MS and the implementation of the first quadrupole as a mass filter provides a very high degree of specificity that enables the selective fragmentation and accurate quantitation of peptides with a wide dynamic range, thus enabling low abundance peptides to be detected against complex backgrounds. This feature is advantageous for proteomic analyses of field-grown leaf tissue, where the high abundance of photosynthetic proteins relative to other cellular components means that any analytical technique must be applicable across a large dynamic range. Secondly, SRM methodologies involve the QqQ MS cycling through a pre-defined list of SRM transitions, which ensures that the abundance of each specified peptide will be measured provided it is present in the sample. This contrasts against the "patchy" nature of shotgun proteomics methodologies, where stochastic elements dictate which proteins are documented in any give run, meaning that proteins of biological interest may escape detection across different runs due to random processes. This particular strength of SRM is well matched to the purpose of biomarker validation studies in crops, where the aim is to assess whether the abundance of a specific protein or proteins is correlated to stress tolerance across a given set of genetic material in real field stress scenarios. The data generated by a controlled environment experiment would thus link the abundance of a particular protein to stress tolerance, and the follow-up experiment would involve profiling the abundance of that protein across a large number of field-grown leaf samples to determine the strength and relevance of the correlation. Thirdly, SRM MS coupled to high-performance liquid chromatography (HPLC) is highly suited to high sample throughput of a wider group or groups of proteins in a single analytical run, thus enabling a larger number of data points on different proteins to be gathered per unit of machine time. This is particularly useful for the assessment of protein changes in field-grown crop plants where the variation in the samples may be larger than those collected in the controlled laboratory environment. This also allows the relationships between changes in the abundance of different proteins to be explored in G × T datasets. Usually within the lab, power analysis will reveal that three to five samples are sufficient for the experiment to be informative and this number is amenable to quantitative shotgun proteomic approaches. However, field analysis may require >20 samples for the experiment to be informative and this would led to prohibitively expensive and time-consuming analysis by shotgun proteomic approaches. In these circumstances SRM MS approaches provide an opportunity to quantitate a select group or groups of proteins from a larger number of samples relativity cheaply and quickly. For instance, research suggest that the proteins involved in reactive oxygen species (ROS) detoxification are strongly linked to abiotic stress tolerance (Gill and Tuteja, 2010), but a full understanding of this link is complicated by the fact that the ROS detoxification network in plants involves many proteins spread across numerous cellular compartments (Mittler et al., 2004). Therefore, it can be argued that a SRM MS approach may aid the investigation of which specific ROS detoxification enzymes are causative in abiotic stress tolerance, as this approach would enable not only a large set of ROS detoxification proteins to be profiled in parallel, but it could also be assessed in a wide range of varieties with differing stress tolerance. This is more likely to reveal which specific components of the large ROS detoxification network exhibit consistently higher abundance values in tolerant genotypes than a shotgun proteomic approach.

DESIRABLE RESOURCES FOR FUTURE SRM MASS SPECTROMETRY STUDIES IN CROP PLANTS

The central difference between SRM MS approaches compared to other proteomics methodologies is the necessity for prior knowledge of proteotypic peptides derived from a protein of interest. Therefore, the most pressing constraint upon SRM approaches in crops is the lack of proteotypic peptides which can be used to quantify proteins of interest in relevant species, meaning that the first step in any SRM investigation involves a long period of library generation, where discovery proteomics and database searching are conducted to define high-quality peptides which are suitable for subsequent SRM experiments. This contrasts with the current state of the SRM field in other species, where for example the SRMAtlas project is collating representative mass spectra for signature peptides derived from tens of thousands of proteins expressed in human, yeast, and mouse (Picotti et al., 2008). This database provides pre-written transition lists for profiling relevant proteins and proteomes (i.e., disease biomarkers in human plasma, central metabolic enzymes in yeast), which can be downloaded and uploaded into the mass spectrometer's control software without the need for in-house optimization. The rate at which SRM MS approaches are applied to crop plants would likely rapidly increase if a publicly available database that provided high-quality proteotypic peptides and optimized multiplexed SRM transition lists to the community was available.

Despite widespread interest in applying molecular techniques to phenotyping crop plants, difficulties in sequencing the large and complex genomes of many crop species have constrained the power of proteomics applied to crops. DNA sequencing technologies are increasing the speed and decreasing the cost of sequencing, with recent highlights being the generation of reference genomes

for barley (Mayer et al., 2012) and tomato (Sato et al., 2012), while rapid progress is being made on the complex wheat genome (Berkman et al., 2012). There is wide variability in abiotic stress tolerance between cultivars (Stone and Nicolas, 1994; James et al., 2008), and different varieties of the same species often exhibit considerable genetic divergence (Gepts, 2006; van de Wouw et al., 2010), so it can be argued that one single "reference" genome per species will not capture the inter-cultivar diversity at the molecular level that manifests in varietal stress tolerance. Studies in wheat cultivars have shown that searching MS results against cultivar-specific sequence databases increases sequence coverage and allows for identification of novel protein isoforms (Altenbach et al., 2010). Therefore, it seems logical that SRM MS studies focusing on varietal differences could derive novel information by profiling the abundance of cultivar-specific protein isoforms, particularly for isoforms of proteins which have been linked to abiotic stress tolerance in different varieties (Sule et al., 2004; Jacoby et al., 2010). A number of initiatives are currently profiling genetic diversity across different varieties of crops (McNally et al., 2009; Lam et al., 2010), and it would be worthwhile for researchers involved in SRM MS studies to develop transitions for the cultivar-specific isoform variants that are documented by these sequencing efforts.

Proteomics research is typically conducted by scientists trained in the disciplines of biochemistry and molecular biology. Their emphasis upon molecular mechanisms is indispensable for understanding the biological meaning of proteomics data. However, due to the specialist knowledge and logistical difficulties inherent in designing and conducting meaningful field experiments, proteomics researchers will likely depend upon collaborations with agriculturally focused researchers in order to access tissue grown in the relevant field environment with appropriate spatial designs and checks. Therefore, building effective collaborations across discipline boundaries is crucial for proteomics researchers who wish to access field-grown material and contribute to crop breeding programs. It can be argued that a mutual appreciation of classical plant physiology is the key bridge between molecular and field researchers, as its emphasis upon dissecting a specific trait at the single plant level is a logical convergence point for ideas and hypotheses stemming from the two different scales (Passioura, 2010). For instance, breeders and agronomists might identify that a given trait (i.e., transpiration efficiency, stem carbohydrate remobilization) leads to a yield improvement under drought conditions, while proteomics researchers can use SRM techniques to investigate the protein abundance profiles of lines which carry this trait, in order to define or validate the proteins that could then serve as candidate genes for breeders. In this way the physiological isolation of a trait which breeders deem to be worthwhile in the target environment can be positioned as a unifying framework which can synthesize the results from molecular studies with agricultural analyses of yield or quality.

CONCLUSION

As claimed by the introductory sections of many papers, as well as preambles to many grant applications, the strategic endpoint of much plant biology research at the molecular scale is to improve the abiotic stress resistance of crop species. However, much of the data produced by plant proteomics research is yet to be actually evaluated for its use in directing breeding programs. Although the initial identification of candidate proteins linked to stress tolerance will still utilize discovery proteomics workflows applied to plants grown under controlled environment conditions in the lab, the technical capabilities of SRM are a better match for validation studies which aim to quantify the abundance of selected target proteins. The ability of the SRM approach to accurately quantify the abundance of a range of target proteins against complex cellular backgrounds in a large number of field-grown samples is a key to its applicability and value. Therefore, we argue that the judicious development of this SRM approach will further our understanding of the causative links between cellular composition and whole plant stress tolerance, and bring the knowledge and skills of proteomics researchers closer to the stated goal of crop improvement for higher yields in harsh environments.

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Proteomic responses of fruits to environmental stresses

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Fruits and vegetables are extremely susceptible to decay and easily lose commercial value after harvest. Different strategies have been developed to control postharvest decay and prevent quality deterioration during postharvest storage, including cold storage, controlled atmosphere (CA), and application of biotic and abiotic stimulus. In this review, mechanisms related to protein level responses of host side and pathogen side were characterized. Protein extraction protocols have been successfully developed for recalcitrant, low protein content fruit tissues. Comparative proteome profiling and functional analysis revealed that defense related proteins, energy metabolism, and antioxidant pathway played important roles in fruits in response to storage conditions and exogenous elicitor treatments. Secretome of pathogenic fungi has been well-investigated and the results indicated that hydrolytic enzymes were the key virulent factors for the pathogen infection. These protein level changes shed new light on interaction among fruits, pathogens, and environmental conditions. Potential postharvest strategies to reduce risk of fruit decay were further proposed based on currently available proteomic data.

Keywords: proteomics, postharvest, fruit, pathogen, induced resistance, secretome

INTRODUCTION

Fruits and vegetables are highly perishable horticultural products, especially during the ripening and postharvest stages, when considerable losses due to microbiological diseases, disorders, transpiration, and senescence can occur. Although quality deterioration of fresh postharvest fruits and vegetables is the result of a number of different factors, microbial activity is by far the single most important one (Sommer, 1982). In the developed countries, approximately 10–30% of harvested fruits and vegetables is lost due to postharvest spoilage, and in the developing countries the losses are over 30–50% annually due to lacking sanitation and refrigeration (Salunkhe et al., 1991; Legard et al., 2000).

The fruit-pathogen interaction depends on mutual recognition. Studies have been performed using classical genetics, cell biology, and biochemistry, as well as high-throughput—omic techniques (Chan and Tian, 2005, 2006; Chan et al., 2007; Qin et al., 2007). As a powerful tool, proteomics approach has been widely used to identify global changes in structure and abundance of plant proteins in response to developmental and environmental signals (Chan et al., 2007; Shi et al., 2008). There has been an increasing trend in application of proteomic methods to detect fruit and vegetable physiological changes over the last few years (Rocco et al., 2006; Chan et al., 2008; Pedreschi et al., 2008), offering to the research community the opportunity to unravel complex sets of proteins.

DEVELOPMENT OF PROTEOMIC PROFILING PROTOCOLS FOR FRUITS AND VEGETABLES

Today, proteomics techniques, including high-resolution two dimensional electrophoresis (2-DE), in-gel proteolytic digestion of protein spots and protein identification by MS through database searches, are increasingly used in studies of animals and microorganisms (Antelmann et al., 1997; Qin et al., 2007), and have been used successfully in plant biology to study changes in protein level expression during development (Chang et al., 2000; Chan et al., 2007). However, plant cells contain many components that may interfere with protein extraction, separation, and purification (Granier, 1988). Fruit tissues are difficult to process through proteomic approach partly due to technical problems such as the low protein content of fruit and the presence of an array of compounds, for example, pigments, starch, polyphenols, polysaccharides, tannins, and organic acids that can cause a high degree of protein denaturation and inactivation (Clements, 1970).

For good reproducibility of 2-DE, sample preparation is a critical step. The extraction of high-quality protein from recalcitrant, low protein content fruit tissue is a challenge. Several protein extraction protocols suitable for 2-DE have been developed. Major soluble proteins of grapevine ripe berries have been extracted from six different cultivars using TCA-acetone solution (TCA method) (Sarry et al., 2004), resulting in three hundreds detected spots on the 2-DE map after colloidal blue staining. Chan and co-authors have developed two additional protocols suitable for fruit protein extraction (Chan et al., 2007, 2008). In one protocol, fruit tissues were homogenized in homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 1% Triton X-100 before TCA-acetone precipitation (Homo method) (Chan et al., 2007). In another protocol, extraction with an equal volume of Tris-HCl pH 7.8 buffered phenol was conducted after buffer homogenization but before methonal precipitation (Phe method) (Chan et al., 2008) (**Figure 1**). In comparison of these three protocols, Homo and Phe methods are much better than TCA method in

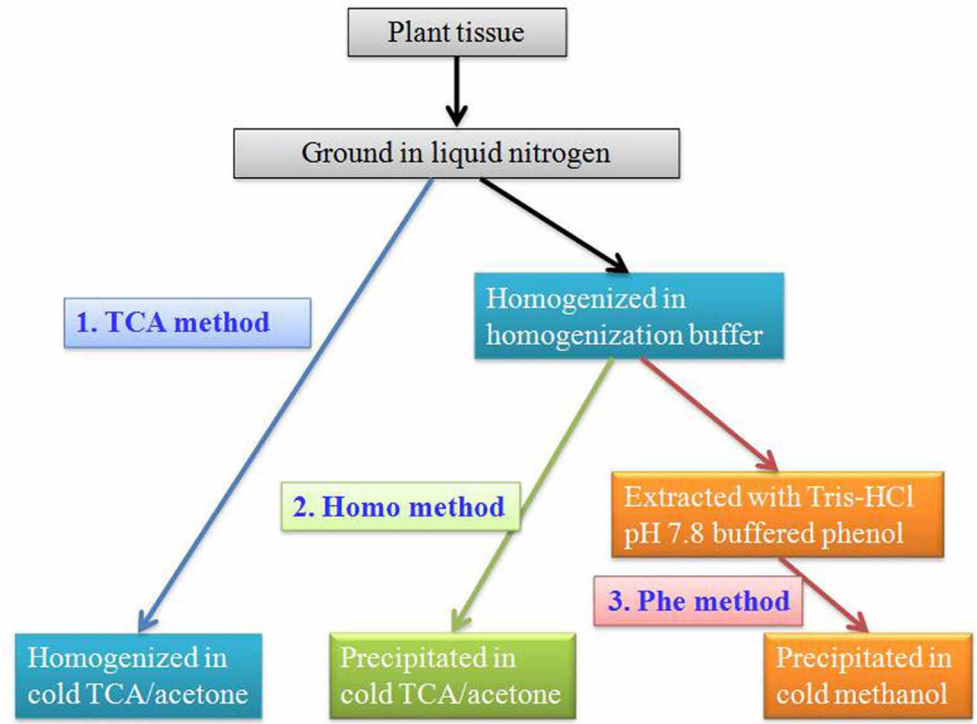

FIGURE 1 | Protein extraction protocols for fruits.

fruit protein extraction. Reproducible 2-DE results with well-resolved polypeptide spots throughout the gel were achieved by Homo and Phe protein extraction protocols for a variety of fruits, including sweet cherry (*Prunus avivum*), peach (*Prunus persica*), apple (*Malus domestica*), mango (*Mangifera indica*), and jujube (*Ziziphus jujuba*) fruits. This method can also be applied as a general method to other plant tissues that are rich in interfering compounds (Wang et al., 2009a).

INTERACTION BETWEEN FRUITS AND FUNGI

As mentioned above, many studies focused on the interactions between fruits and pathogens based on proteomics approach. However, the major problem is how to identify proteins from fruit-pathogen mixed samples. Therefore, few proteomic data are available for fruit (host) side and also for pathogen side *in vivo*. One possibility is that specific taxon should be used when mass spectrometry data were submitted to the website of Matrix Science for protein identification. After searching using both host taxon and pathogen taxon, we are able to tell where the proteins come from. Gray mold by *Botrytis cinerea* and green mold by *Penicillium expansum*, two major diseases caused enormous fruit decay and great economic losses, have been extensively studied by several research groups and part of the results were reviewed here.

Botrytis cinerea

B. cinerea is a pathogenic filamentous fungus which infects more than 200 plant species in a variety of organs including fruit, flowers, and leaves (Williamson et al., 1995; Fernández-Acero et al., 2011). The host range for *B. cinerea* infection includes economically important crops such as tomato, berries, chickpeas, french beans, and cut flowers as well as many fruits. The fungus causes the gray mold disease resulting in significant crop losses under different production conditions. Gray mold occurs over a wide geographical area, in the open field, in greenhouses and even in storages at 0–10°C. *B. cinerea* is the principal cause of pre- and post-harvest disease in grapes, berries, tomatoes, and many other crops (Elad, 1997; Williamson et al., 2007). Proteomic analysis of three types of tomato fruit infected by *B. cinerea* revealed that 186 tomato proteins were identified in common among red ripe and red ripe-equivalent ripening inhibited (rin) mutant tomato fruit infected by *B. cinerea*. However, the limited infections by *B. cinerea* of mature green wild type fruit resulted in 25 and 33% fewer defense-related tomato proteins than in red and rin fruit, respectively (Shah et al., 2012).

The biology of *B. cinerea* has been studied extensively and the genome of the fungus has been sequenced (Elad et al., 2004; van Kan, 2006), including strain T4 (INRA/Genoscope: http://urgi.versailles.inra.fr/projects/Botrytis/) and strain B05.10 (Broad Institute, Massachusetts Institute of Technology, MIT, http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html). *B. cinerea* secretes a battery of enzymes utilized for the degradation and consumption of the host plant. In 2006, Fernández-Acero et al. reported the first approach to the proteome analysis of *B. cinerea*. Most of the identified spots may play a crucial role as pathogenicity or virulence factors, including some housekeeping enzymes, such as malate and glyceraldehyde dehydrogenases. In an attempt to identify putative fungal virulence factors, protein profile from two *B. cinerea* strains differing in virulence and toxin production were compared. Twenty seven

protein spots were identified and a significant number of spots were identified as malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, which could be ascribed to differences in virulence between strains (Fernández-Acero et al., 2007). Shotgun proteomics was successfully used to identify the secretome of *B. cinerea* grown in three culture conditions which differed by the carbon nutrients provided. A total of 126 proteins secreted by *B. cinerea* were identified, 13 of which were identified as pectinases, which play an important role in cell wall degradation and successful invasion (Shah et al., 2009). Furthermore, the effect of ambient pH on secretome of *B. cinerea* strain B05.10 was investigated with a comparative proteomic method. The results indicated that distinct differences in secretome of *B. cinerea* were found between pH 4 and 6 treatments, and 47 differential spots, corresponding to 21 unique proteins, were identified. At pH 4, more proteins related to proteolysis were induced, whereas most of up-accumulated proteins were cell wall degrading enzymes at pH 6 (Li et al., 2012).

Penicillium expansum

P. expansum, another widespread filamentous fungus, is a major causative agent of fruit decay with great economic losses. This strain is also of potential public health concern, because it produces toxic secondary metabolites, including patulin, citrinin, and chaetoglobosins (Andersen et al., 2004).

In a study to compare the cellular and extracellular proteomes of *P. expansum*, the results showed that several proteins related to stress response (glutathione S-transferase, catalase, and heat shock protein 60) and basic metabolism (glyceraldehyde-3-phosphate dehydrogenase, dihydroxy-acid dehydratase, and arginase) were identified in the cellular proteome. Catalase and glutathione S-transferase, the two antioxidant enzymes, exhibited reduced levels of expression upon exposure to borate, which affects the virulence of the fungal pathogen. The extracellular proteome of *P. expansum* under stress condition with reduced virulence showed that the expression of three protein spots were repressed in the presence of borate and identified as the same hydrolytic enzyme, polygalacturonase (Qin et al., 2007).

Exogenous environmental conditions greatly affect infection of pathogens, including temperature, humility, pH value, and oxidative status. H_2O_2 is reported to have a direct antimicrobial effect and be involved in defense reactions activated in plant tissues upon pathogen attack (Mellersh et al., 2002). Plasma membrane damage was not the main reason for H_2O_2-induced death of the fungal pathogen. Proteomic analysis of the changes of total cellular proteins in *P. expansum* showed that a large proportion of the differentially expressed proteins appeared to be of mitochondrial origin, implying that mitochondria may be involved in this process. Further mitochondrial sub-proteomic analysis characterized a set of mitochondrial proteins, including respiratory chain complexes I and III, F_1F_0 ATP synthase, and mitochondrial phosphate carrier protein which might be associated with fungal death caused by H_2O_2 (Qin et al., 2011). The pH value, as one of the most important environmental parameters, has critical influence on spore germination of *P. expansum*. Spore germination of *P. expansum* was obviously inhibited at pH 2.0 and 8.0. Comparative proteomics analysis revealed that 17 proteins involved in protein synthesis and folding were mainly down-regulated at pH 2.0 and 8.0. These findings indicated that impairing synthesis and folding of proteins might be one of the main reasons account for ambient pH effect on spore germination of *P. expansum* (Li et al., 2010).

INDUCED RESISTANCE (IR) OF FRUITS

Traditionally, postharvest disease is often controlled by the application of synthetic fungicides (Eckert and Ogawa, 1988). However, chemical protection is discouraged due to problems related to fungicide toxicity, development of fungicide resistance by pathogens, and potential harmful effects on the environment and human health, alternatives to synthetic chemicals have been proposed (Eckert et al., 1994; Tian and Fan, 2000; Elad et al., 2004). The use of biologically based fungicides in conjunction with induced resistance (IR) was suggested as a feasible approach for reducing postharvest disease in harvested fruits and vegetables (Cook et al., 1999; Tian et al., 2001). IR is a plastic response, which diverts carbon and nitrogen resources from plant growth and reproduction to provide a long lasting and systemic resistance to a broad spectrum of pathogens and pests (Linda, 2001). Two types of IR are well-characterized. Systemic acquired resistance (SAR) is an active defense initiated by infection with certain necrotizing pathogens and confers resistance to secondary infection. SAR is effective against a broad-spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Ryals et al., 1996; Sticher et al., 1997). Inhibition of salicylic acid (SA) accumulation or biosynthesis impairs SAR (Gaffney et al., 1993). Induced systemic resistance (ISR) resembles SAR but is induced by root colonization of specific strains of non-pathogenic plant growth-promoting rhizobacteria in contrast to SAR that is induced by necrotizing pathogens. Unlike SAR, ISR is dependent on jasmonate and ethylene, independent on SA and not associated with *PR* gene expression (van Loon, 1997). Molecularly, both SAR and ISR in Arabidopsis are intertwined through *NPR1* gene (**Figure 2**).

In fruits, IR can be triggered by microbial biological agents (non-pathogens, avirulent forms of pathogens), physical agents (curing, γ-radiation, hot water brushing and UV-C light), certain chemical agents [DL-3-amino butyric acid (BABA), 1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester (ASM), salicylic acid (SA), ethylene, harpin, 2,6-dichloroisonicotinic acid, jasmonic acid (JA), methyl jasmonate (MJ), Oxalic acid (OA), potassium and phosphates], and natural compounds (Chitosan and Margosan-O) (Tian and Chan, 2004). In many experiments, IR holds promise as a new technology for the control of postharvest diseases and has been proven to be effective in the laboratory and in a few field cases (Droby et al., 2001; El-Ghaouth et al., 2003; Chan et al., 2008). Mechanisms of IR have been well-characterized from cell structure, physiological, and biochemical changes. Proteomic studies shed a light on molecular changes of IR.

MICROBIAL BIOLOGICAL AGENTS

In recent years, considerable attention has been placed on postharvest application of antagonists for the inhibition of plant disease because of concerns about the application of

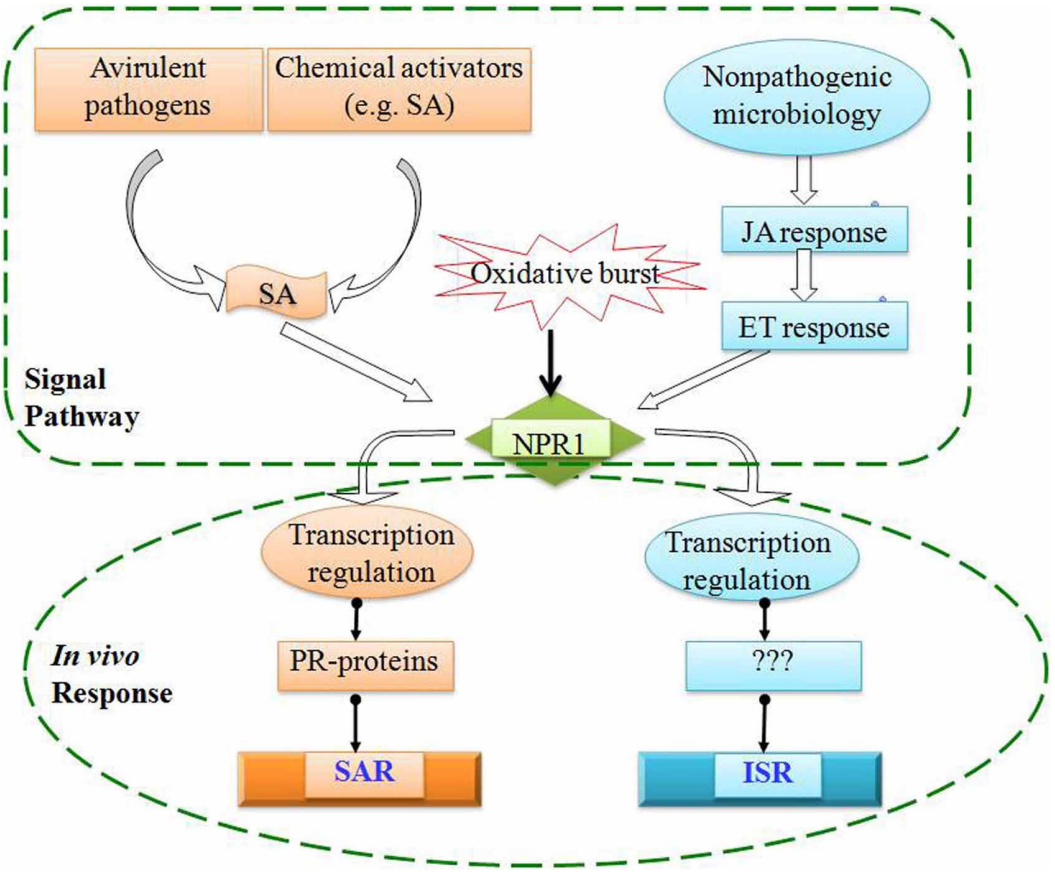

FIGURE 2 | Signal transduction pathways for systemic acquired resistance (SAR) and induced systemic resistance (ISR).

synthetic chemicals. Utilization of antagonistic yeasts as an alternative appears to be a promising technology (Droby et al., 2001; El-Ghaouth et al., 2003; Chan and Tian, 2006). Some antagonist-based products are commercially available and others are currently under varying degrees of development (Castoria et al., 2001).

Several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts, including direct interaction between antagonistic yeasts and pathogens (El-Ghaouth et al., 2003; Chan and Tian, 2005) and IR of fruit tissues by antagonistic yeasts (Droby et al., 2001; Chan and Tian, 2006; Chan et al., 2007). Significant changes in polyphenoloxidase (PPO), peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and phenylalanine ammonia-lyase (PAL) activities were found to be involved in the IR after antagonistic yeasts treatment (Fan and Tian, 2000; Qin et al., 2002; Chan and Tian, 2006). In harvested peach fruit, 19 proteins were identified using quadrupole time of-flight tandem mass spectrometer after treatment with antagonistic *Pichia membranefaciens*, including antioxidant related proteins, stress responsive proteins and proteins involved in energy pathways (Chan et al., 2007). These results suggested that antioxidant and PR proteins, as well as enzymes associated with sugar metabolism, were involved in resistance of peach fruit induced by *P. membranefaciens*.

HEAT TREATMENT

Pre-storage heat treatment is a promising postharvest method for reducing disease incidence and severity (Terry and Joyce, 2004; Schirra et al., 2011). Pre- and post-harvest heat treatments increased the resistance of cherry tomato (Zhao et al., 2009), bamboo shoots (Luo et al., 2012), fresh cut broccoli (Moreira Mdel et al., 2011), strawberry (Marquenie et al., 2002; Villa-Rojas et al., 2011), lemon (Nafussi et al., 2001) against postharvest decay, and reduced the development of green mold on citrus fruit (Schirra et al., 2008a), blue mold on pear (Schirra et al., 2008b), and peach (Zhang et al., 2007).

Based on the 2D-DIGE analysis, 52 proteins were differentially expressed between peach fruits exposed to heat treatment or after transfer to 20°C, vs. fruits kept at 20°C. Among identified spots, a large number (93%) have been proposed to play a role in plant metabolism such as the defense and stress response, cytoskeleton organization, primary metabolism, transcription and translation regulation, and protein storage and catabolism. The category of proteins participating in biotic or abiotic stress responses was the one with the most proteins differentially expressed. Additionally, one third of the identified proteins corresponded to the large family of HSPs and exhibited molecular masses <20 kDa, indicating that the induction of small HSPs in heated peach may participate in the acquisition of tolerance against some chilling

injury symptoms (Lara et al., 2009). Another study demonstrated that among the thirty protein spots in peach fruit induced by heat treatment, 43% were related to stress response, 17% to cell structure, 13% to protein fate, 7% to glycolytic pathway, 3% to ripening and senescence, and 17% to unclassified (Zhang et al., 2011). The commonly induced proteins from both groups included ascorbate peroxidase, heat shock proteins, and allergen proteins, indicating these proteins are heat inducible proteins in peach fruit.

In citrus fruit, heat treatment induces defense mechanisms and triggers physiological responses to withstand stressful conditions during storage. Functional classification of twenty eight differentially expressed proteins showed that the main affected categories were "Cell rescue, defense, and virulence" and "Metabolism." Activity of antioxidant enzymes was extensively changed upon heat treatment, including SOD, POD and alcohol dehydrogenase (Perotti et al., 2011).

CHEMICAL AGENTS

Certain chemicals, including SA and OA, emerged as potentially effective agents to IR in fruits. SA has emerged as a key signaling component involved in the activation of certain plant defense responses (Durner et al., 1997). Exogenous application of SA protects plants against certain pathogens and activates SAR in a wide variety of plant species, including harvested fruit (van Loon, 1997; Yao and Tian, 2005). SA treatment IR to MYMIV infection in *Vigna mungo*. Twenty-nine proteins identified by MALDI-TOF/TOF, predicted to be involved in stress responses, metabolism, photosynthesis, transport, and signal transduction, showed increased abundance upon SA treatment. SA treatment stimulated SOD and POD activity and inhibited CAT activity thus preventing ROS mediated damage (Kundu et al., 2011). In harvested peach fruit, thirteen SA induced proteins were identified and the functions of these proteins were mainly involved in antioxidant and energy pathway (Chan et al., 2007). Pre-harvest application of SA solution enhanced the resistance of sweet cherry fruits against *P. expansum*, resulting in lower disease incidences and smaller lesion diameters, especially at earlier maturity stage. Totally 13 and 28 proteins were identified after SA treatment at earlier (A) and later (B) maturity stage, respectively. Antioxidant proteins and pathogenesis related-proteins were identified at both A and B stages, while heat shock proteins and dehydrogenases involved in glycolysis and tricarboxylic acid cycle were only detected at B stage (Chan et al., 2008). All these results indicated that antioxidant related proteins as well as proteins involved energy metabolism play key roles in SA induced resistance in fruits.

OA is an organic acid distributing widely in various organisms, especially in plants (Franceschi and Nakata, 2005). Recent studies revealed that OA might play important roles in systemic resistance, stress response, programmed cell death and redox homeostasis in plant (Guo et al., 2005; Kim et al., 2008). Recent studies showed that application of OA extend postharvest and shelf life in litchi (Zheng and Tian, 2006), mango (Zheng et al., 2007a), peach (Zheng et al., 2007b), jujube (Wang et al., 2009b), and plum (Wu et al., 2011) fruits. The application of oxalic acid reduced ethylene production and delayed softening of plum fruit. During storage or shelf-life, flesh reddening and anthocyanin synthesis were significantly inhibited in oxalic acid-treated plum fruit, accompanied with decreased PAL activity (Wu et al., 2011). In jujube fruit, application of OA at the concentration of 5 mM could delay fruit senescence by reducing ethylene production, repressing fruit reddening and reducing alcohol content, which consequently increased fruit resistance against blue mold caused by *P. expansum*. A total of 25 differentially expressed proteins were identified after OA treatment. The activity of alcohol dehydrogenase 1 and 1-aminocyclopropane-1-carboxylic acid synthase was repressed, while the abundances of three photosynthesis-related proteins, a cystathionine β-synthase domain-containing protein and three proteins related to the defense/stress response were up-regulated by OA. These results indicated that OA treatment might affect ethanol and ethylene metabolism, resulting in delaying senescence, and increase resistance of jujube fruits against fungal pathogens (Wang et al., 2009b).

PROTEOME-LEVEL RESPONSES OF FRUITS TO STORAGE ENVIRONMENTS

COLD STORAGE

Cold storage is one of the most frequently employed methods to delay fruit senescence and maintain fruit quality during postharvest storage. Generally speaking, fruits taste good with a total soluble sugar/total organic acid ratio over 8 and low temperature (LT) is applied in inhibiting the ratio increase (Yun et al., 2012). Under LT condition, a total of 108 differentially accumulated protein spots in citrus were analyzed by MALDI-TOF MS/MS, and 63 spots were successfully identified on the basis of tryptic peptide sequences. The results showed that metabolism pathway and stress response related categories were considerably enriched, including sugars and polysaccharide metabolism, secondary metabolism, protein destination and storage, and response to stimulus (Yun et al., 2012). Moreover, fruit maintains low accumulation of ROS, low level of membrane lipid peroxidation, greater flesh firmness and higher concentrations of organic acids and, vitamin levels (Kan et al., 2011; Yun et al., 2012). These results indicated that these processes play a leading role in the maintenance of fruit quality and delaying of senescence at LT.

Interestingly, peach fruit is less prone to chilling injury when stored at 0°C than at 5°C (Zhang et al., 2010). Further study using proteomics approach indicated that several membrane stability related proteins were enhanced, while proteins related to phenolic compounds metabolization were repressed in peach fruit at 0°C relative to 5°C. Other proteins involved in sugar metabolism and energy pathways were decreased at 0°C, resulting in the lower assumption of sugars which has several beneficial effects in protecting plants against stresses (Zhang et al., 2010). When stored at LTs, 55 proteins from bell pepper (*Capsicum annuum*) fruits were identified as having differential abundances. Redox and carbohydrate metabolism were the two major functional classes found in a proteomic study (Sánchez-Bel et al., 2012).

Dehydrin is known as a group 2 late embryogenesis abundant (LEA) proteins, one of several ubiquitous water-stress-responsive proteins in plants (Close, 1997). Several individual groups identified dehydrin protein in peach and citrus fruit stored

at LT through traditional 2D-PAGE and difference gel electrophoresis (DIGE) (Nilo et al., 2010; Yun et al., 2010, 2012; Zhang et al., 2010). Other commonly identified proteins by these groups included heat shock protein and dehydrogenase which were well-known to play a key role in plant stress responses (Giannino et al., 2004; Posner et al., 2012). In nectarine fruits stored at LT, four differentially expressed proteins were characterized as allergens which can provide some form of protection to fruits during periods of stress (Pedreschi et al., 2007; Giraldo et al., 2012).

CONTROLLED ATMOSPHERE (CA)

Fruit are often stored under controlled atmosphere (CA) conditions with low-O_2 and high-CO_2 at LT to reduce respiration, ethylene production rates and action, delay ripening and senescence, as well as to reduce the growth of pathogens (Kader, 2002). When loquat (*Eriobotrya japonica* Lindl.) fruit were stored under different storage conditions, CA with 10% O_2 + 1%CO_2 was more effective in reducing fruit decay. Loquat fruit could be stored in this CA condition at 1°C for more than 50 d with normal flavor and low decay index of about 7%. CA conditions were more effective in reducing the activities of PPO and oxidative stress compared to other treatments (Ding et al., 2006). The same results were achieved that CA conditions were more effective in reducing total phenol content, delaying anthocyanidin decomposition, preventing pericarp browning, and decreasing Litchi (*Litchi chinensis* Sonn. cv Heiye) fruit decay in comparison with other treatments (Tian et al., 2005a). Proteomic analysis revealed that several proteins were significantly changed in pear during CA conditions. Triosephosphate isomerase, a key enzyme of the energy metabolism was up-regulated under browning-inducing conditions. ACC oxidase involved in ethylene biosynthesis and the major allergen Pyrc 1 were clearly down-regulated under low oxygen or high carbon dioxide concentrations. Stress responsive proteins, like the chaperone molecule HSP70, were also down-regulated as the oxygen concentration diminished (Pedreschi et al., 2008).

PROTEOME-LEVEL CHANGES DURING FRUIT DEVELOPMENT AND RIPENING

Fruit ripening is a developmental complex process which occurs in higher plants and involves a number of stages displayed from immature to mature fruits that depend on the plant species and the environmental conditions (Palma et al., 2011). The pathways involved in the processes of fruit development and ripening are exclusive for plants and vary between species. Based on ethylene production, two types of fruits were characterized, i.e., either climateric or non-climateric. Climacteric fruits, such as tomato, papaya, peach, apple, pear, banana, plum, and melon, are characterized by a dramatic increase in ethylene production, which is responsible for the typical respiratory burst during ripening, and the activation of many biochemical steps (Barry and Giovannoni, 2007; Palma et al., 2011). On the other hand, ripening of non-climateric fruits such as pepper, citrus, orange, cucumber, grape, cherry, and strawberry is ethylene-independent (Palma et al., 2011).

CLIMATERIC FRUITS

In young tomato fruit, intensity of proteins involved in amino acid metabolism and protein synthesis increased during the early development (cell division) stage. During the later development (cell expansion) stage, proteins functioning in photosynthesis and cell wall formation transiently increased. In contrast, many proteins related to C compounds and carbohydrate metabolism or oxidative processes were up-regulated during fruit development (Faurobert et al., 2007). In papaya, six main categories, including cell wall, ethylene biosynthesis, climacteric respiratory burst, stress response, synthesis of carotenoid precursors, and chromoplast differentiation were found to be related to fruit ripening using 2DE-DIGE (Nogueira et al., 2012). Based on traditional 2D-PAGE, several cell wall degrading enzymes related to fruit ripening were identified in papaya fruit (Huerta-Ocampo et al., 2012). In peach fruit, the functions of 30 identified proteins were involved in primary metabolism (e.g., C-compounds, carbohydrates, organic acids, and amino acids) and in ethylene biosynthesis as well as proteins involved in secondary metabolism and responses to stress (Prinsi et al., 2011). A further study to characterized the protein accumulation patterns in firm and soft fruit of three peach and two nectarine melting flesh varieties revealed that 164 of the 621 protein spots analyzed displayed a differential accumulation associated with the softening process. Among them, only 14 proteins changed their accumulation in all the varieties assessed, including proteins mostly involved in carbohydrates and cell wall metabolism as well as fruit senescence (Nilo et al., 2012). Moreover, in kiwifruit, which displays climacteric behavior at temperatures above 20°C (Antunes, 2007), proteomic analysis using 1D-SDS-PAGE and mass spectrometry identified 102 kiwifruit proteins during ripening, which are mainly involved in energy, protein metabolism, defense, and cell structure. Ripening induced protein carbonylation in kiwifruit but this effect was depressed by ozone (Minas et al., 2012).

NON-CLIMATERIC FRUITS

The proteins identified as differentially accumulated during ripening of strawberry fruit are involved in a wide range of biological processes such as energy and carbon metabolisms, secondary metabolism/biosynthesis of cellular components, cellular organization, communication and signal transduction, protein metabolism, stress response, and transcription. Most of identified proteins showed a regular increase in spot volume from the immature to the mature stage indicating they are progressively involved during ripening (Bianco et al., 2009). Grape is another non-climateric important crop species for human nutrition and agricultural economy. The proteomic analysis using grape skin tissue revealed that the most relevant changes in protein expression occurred in the first weeks of ripening. Many of these variations were related to proteins involved in responses to stress, glycolysis and gluconeogenesis, C-compounds and carbohydrate metabolism, and amino acid metabolism (Negri et al., 2008). Another study revealed that proteins involved in photosynthesis, carbohydrate metabolisms, and stress response are identified as being enriched at the beginning of color-change. The end of color-change is characterized by the enrichment of proteins involved in anthocyanin synthesis and, at harvest, the

dominant proteins are involved in defense mechanisms. In particular, the abundance of different chitinase and beta-1,3-glucanase isoforms increased as the berry ripens, indicating these enzymes were involved in softening during fruit ripening (Deytieux et al., 2007).

TOOLS FOR FRUIT PROTEOME PROFILING

Traditional large scale proteomic analysis usually relies on 2D gel electrophoresis (2DE), i.e., separation by 2DE, protein spot digestion with a protease, and MS identification. However, gel electrophoresis is poorly compatible with high-throughput MS analysis. More recently, gel free technology has been well-developed, which includes steps of protease digestion of the protein mix, LC separation of peptides, and MS identification. Though the 2DE is still the most commonly used method, gel free based protein separation approaches, like MudPIT (multidimensional protein identification technology), DIGE (2D-difference gel electrophoresis), iTRAQ (Isobaric tag for relative and absolute quantitation), SILAC (stable isotope labeling by amino acids in cell culture), ICAT (isotope coded affinity tags), and LOPIT (localization of organelle proteins by isotope tagging), are potentially powerful tools for fruit proteomes analyses.

2D-DIGE is a powerful technique for quantitatively comparing different samples labeled with different dyes. Using this technology, 37 proteins showing different abundance during papaya fruit ripening were characterized and submitted to MS analysis (Nogueira et al., 2012). In addition, approximately 2500 protein spots were successfully resolved from ripening strawberry fruit 2D-DIGE gel (Bianco et al., 2009). In iTRAQ, peptides derived from each sample are derivatized with amine-specific isobaric tags which are indistinguishable by MS but exhibit MS/MS signature ions. A total of 1664 proteins were identified from orange by the iTRAQ technique (Ai et al., 2012). Researchers also successfully identified genetically engineered tomato using iTRAQ and MudPIT methods (Robertson et al., 2012). These non-gel based approaches are complementary to gel-based ones and together the different techniques allow for improved proteomic coverage (Quirino et al., 2010).

CONCLUDING REMARKS AND FUTURE PROSPECT

Proteomics approach is not only a powerful tool to dissect fundamental level changes, but for selection of appropriate markers (proteins) so as to be able to detect metabolic disorders in harvested fruits at an early stage and identify virulent factors involved in pathogen infection. Based on proteomic studies, proteins involved in different metabolic pathways in fruit were activated after postharvest treatments. Therefore, to reduce postharvest decay and thus minimize ecological losses, biologists should firstly pay more attentions to combination treatments. Several pioneering works have been

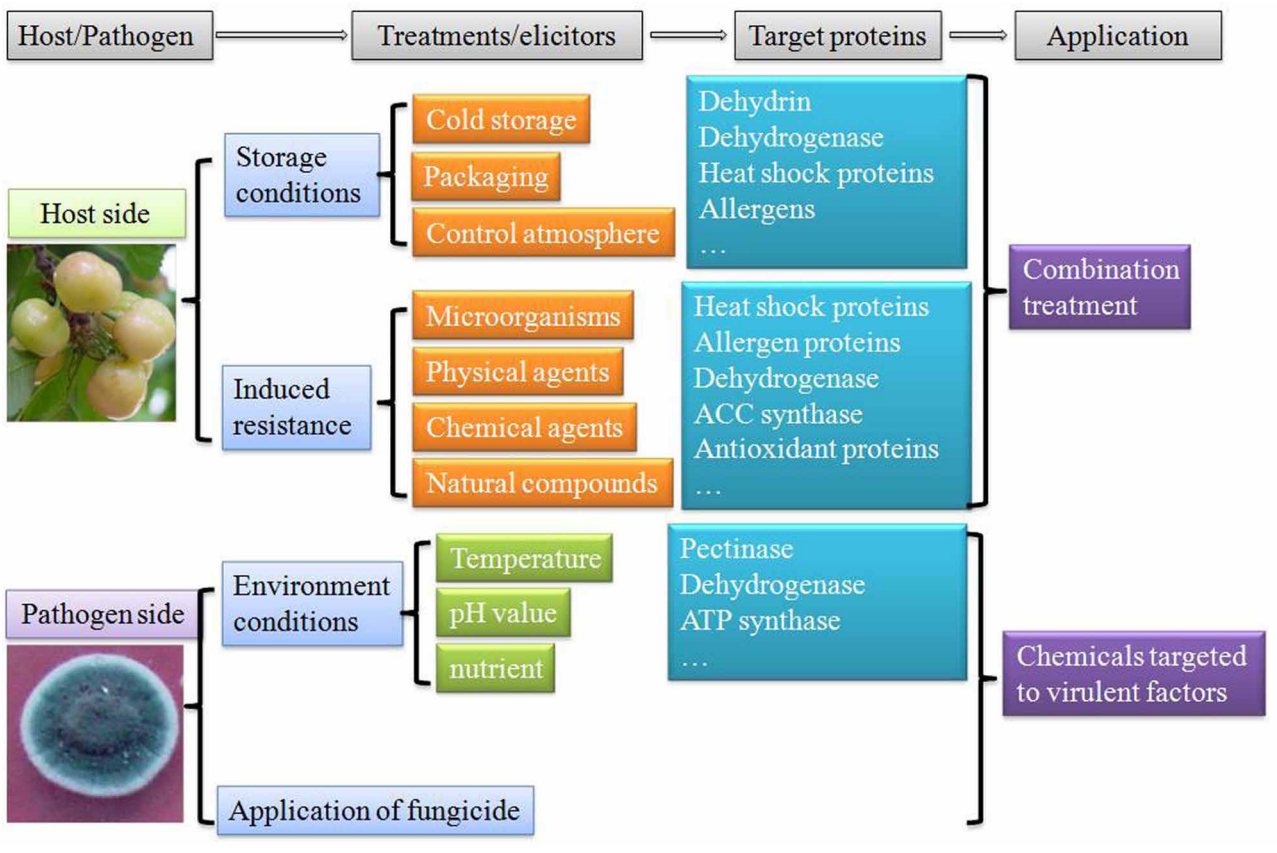

FIGURE 3 | Approaches to control postharvest decay and mechanisms involved based on proteomic study.

carried out to control postharvest diseases by combining different approaches (Tian et al., 2005b; Palou et al., 2007; Sivakumar et al., 2008; Wang et al., 2011). Combination treatments showed improved control efficiency to reduce fruit disorder and decay, and maintain fruit quality during postharvest storage and transportation. Moreover, proteomic approach from the pathogen side also revealed that virulent factors play key roles during pathogen infection. Specific medicines targeted to these proteins could be designed to inhibit the development and growth of pathogens (**Figure 3**). Through these two strategies the biologists were able to more effectively protect the fruit from the infection of postharvest pathogens. Further researches combining proteomic, transcriptomic, and metabolic approaches are also needed to characterize mechanisms involved in interactions among fruits, pathogens and environmental conditions.

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Proteomic studies of the abiotic stresses response in model moss – *Physcomitrella patens*

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Moss species *Physcomitrella patens* has been used as a model system in plant science for several years, because it has a short life cycle and is easy to be handled. With the completion of its genome sequencing, more and more proteomic analyses were conducted to study the mechanisms of *P. patens* abiotic stress resistance. It can be concluded from these studies that abiotic stresses could lead to the repression of photosynthesis and enhancement of respiration in *P. patens*, although different stresses could also result in specific responses. Comparative analysis showed that the responses to drought and salinity were very similar to that of abscisic acid, while the response to cold was quite different from these three. Based on previous studies, it is proposed that sub-proteomic studies on organelles or protein modifications, as well as functional characterization of those candidate proteins identified from proteomic studies will help us to further understand the mechanisms of abiotic stress resistance in *P. patens*.

Keywords: *Physcomitrella patens*, abiotic stress, proteomics

INTRODUCTION

Being sessile, plants are continuously exposed to different biotic and abiotic stresses. The abiotic stress factors, which include drought, salinity, extreme temperature (cold and high temperature), heavy metal, and so on, are becoming more and more serious to the agricultural production all over the world. During the long evolutionary history, plants have evolved the ability to survive the adverse effects of stresses. Investigation of stress-responsive mechanisms has been a major topic for several decades in plant biology. Upon the reception of stress signal, plants could initiate a series of signal transduction, which triggers some protective responses to ensure a survival. Previously, it has been shown that stresses could result in the increasing of reactive oxygen species (ROS; Allen et al., 2000), cytosolic Ca^{2+} concentration (Apel and Hirt, 2004) and some other compounds that might function as secondary messengers and regulate downstream events such as protein phosphorylation and transportation (Halfter et al., 2000). The whole signaling process will lead to changes in the expression of stress-responsive genes. In recent years, substantial progress has been made through the study of model plants, e.g., *Arabidopsis thaliana* and *Oryza sativa*. Many genes that play critical roles in abiotic stress response have been widely studied, especially some transcription factor encoding genes. Among the abiotic stress regulated genes, some are responsible for the biosynthesis of abscisic acid (ABA; Rabbani et al., 2003). ABA is involved in many aspects of plant growth and development including the adaptation to abiotic stresses (Wasilewska et al., 2008). Overlapping between the ABA and abiotic stresses signaling pathways indicate that the abiotic stress responses in plants are at least partially mediated by the ABA signal transduction (Ishitani et al., 1997).

Evidence at the phylogenetic and paleobotanic aspects implies that the variety of terrestrial land plants evolved from a single colonization of the land about 480 million years ago (Kenrick and Crane, 1997). Except for the uncertainty of water supply, the land plants also had to confront with different environmental stresses, such as radiation and extreme temperature, when they first colonized a terrestrial habitat. The strategy for plant to deal with the terrestrial environment includes both the anatomical adaptation and the physiological and biochemical adjustments (Oliver et al., 2005). Most of the vascular plants have evolved some anatomical adaptation mechanisms. In contrast to the complex plants, the less complex plants have very simple anatomical structure. The stresses might directly work on individual cells in these plants. They adapt to environmental stress through biochemical adjustments at the cellular level. Based on the current knowledge, it is known that there are both common and species specific abiotic stress response mechanisms in plant kingdom. So it is necessary to study both complex and less complex plants in order to get a comprehensive idea about the abiotic stress response mechanisms.

Bryophytes, which comprise hornworts, mosses, and liverworts, are placed in a phylogenetic position between the green algae and the seed plants. It is believed that the ancestors of mosses and seed plants separated shortly after the transition from water to land at least 500 million years ago (Heckman et al., 2001; Hedges et al., 2004). They have very simple structures, which makes them ideal for studying the biochemical adjustment in response to abiotic stress. Recently, the moss species *Physcomitrella patens* has been getting more and more attention as a model plant for studying the unknown functions of genes as well as the response mechanisms to different abiotic stresses. This was mainly because of its characteristics which include simple cell structure and efficient homolog recombination (Schaefer and Zryd, 1997). In addition, the *P. patens* is the first bryophyte whose genome has

been sequenced (Rensing et al., 2008). Its genome size is ~480 Mb, similar with that of the rice.

With the availability of genome information for different plant species, functional genomic approaches have been widely applied in the study of abiotic stresses resistance mechanisms in plants (Seki et al., 2001; Chen et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Provart et al., 2003; Rabbani et al., 2003; Zeller et al., 2009). In the last two decades, proteomics has been shown to be a powerful tool in exploring many biological mechanisms at the systematic level. A lot of proteomic analyses have been conducted in *Arabidopsis* (Jiang et al., 2007) and rice (Yan et al., 2006; Li et al., 2010), which brought much deeper insight in the abiotic stress-responsive mechanisms. However, the proteomic studies in another model plant – *P. patens* are still very limited. In recent years, some proteomic studies including both profiling and phosphoproteomic analyses have been carried out in this moss species. Here, we summarized those studies in order to comprehensively understand the mechanisms of abiotic stress response in *P. patens* and get a clear idea for future work.

COMMON RESPONSES AMONG DIFFERENT STRESSES

As indicated previously, overlapping is a common phenomenon among the genes regulated by different abiotic stresses (Ishitani et al., 1997). In our recent studies (Wang et al., 2008, 2009a,b, 2010), when different abiotic stresses including dehydration, salt, cold, and ABA treatment were applied on *P. patens*, a lot of proteins were regulated coordinately. Comparative analyses showed that considerable overlapping existed among the proteins that were regulated by these four different stresses. Specifically, 2 were regulated by all four treatments; 12 were regulated by dehydration, salt, and ABA; 2 by dehydration, salt, and cold, 2 by dehydration, cold, and ABA; and 3 by salt, cold, and ABA. Thirty-four were regulated by two of the treatments (**Figure 1**; **Table 1**). Among all the differentially displayed proteins, 27, 24, 27, and 19 were regulated exclusively by dehydration, salt, cold, and ABA treatment, respectively (**Figure 1**). Based on these comparisons, it could be proposed that the relationship among the dehydration, salt, and ABA treatments is closer than that between anyone of these three with cold treatment, which has also been shown in rice and *Arabidopsis* at both transcriptomic (Seki et al., 2002; Rabbani et al., 2003) and proteomic levels (Li et al., 2010). It has been suggested that ABA is involved in plants responses to environmental stresses, particularly drought and salinity (Zhu, 2002). In *P. patens*, pretreatment with ABA could enhance its desiccation and freezing tolerance (Khandelwal et al., 2010; Richardt et al., 2010). The data in *P. patens* implies that the involvement of ABA in abiotic stress response might have been evolved prior to the divergence of moss and vascular plants during evolution.

Among all the stress-responsive proteins, the energy and metabolism related proteins were the largest group that was commonly regulated by different treatments. They accounted for ~29, 35, 41, and 29 of the total changed proteins in the treatment of salinity, dehydration, cold, and ABA, respectively. Generally, the anabolic proteins were decreased while the catabolic proteins were increased by the stresses (Wang et al., 2008, 2009a,b, 2010). The expressional pattern of the proteins in this group indicated that the respiration was enhanced while the photosynthesis was

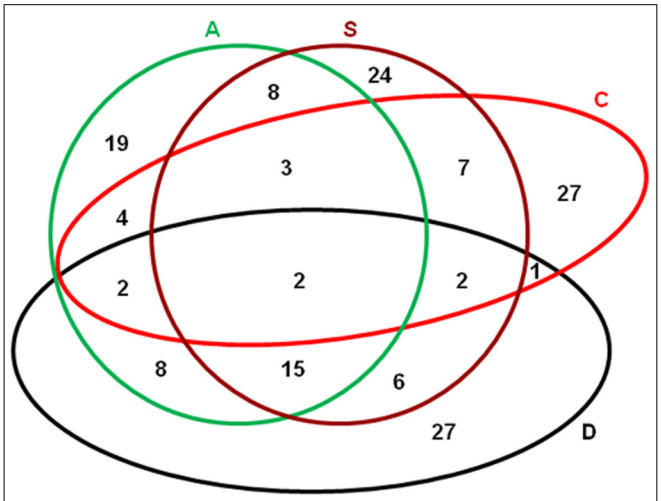

FIGURE 1 | Venn map showing the proteins that were regulated by different abiotic stresses and ABA treatments. D, desiccation treatment: dehydrating to 90% fresh weight loss; S, salt treatment: >0.25 M NaCl for 3 days; C, cold treatment: 0°C incubation for >1 day; A, 50 μM ABA treat for 3 days. The raw data for this figure were from our previous studies (Wang et al., 2008, 2009a,b, 2010).

repressed by the abiotic stresses, although most of the treatments (not including dehydration) did not result in the inhibition of *P. patens* growth (Wang et al., 2008, 2009a,b, 2010). The repression of photosynthesis and enhancement of respiration seem to be a common response to the abiotic stresses in the plant kingdom. Further proteomics studies to the chloroplast and mitochondria might be very helpful to deeply understand the molecular mechanisms of *P. patens* resistance to different abiotic stresses.

In addition to similar changes of the energy and metabolism associated protein, some defense related proteins, such as APX, peroxiredoxin, and heat shock proteins (HSPs), were also changed in a similar way in responding to various abiotic stresses. Specifically, different members of HSP70s were dramatically changed upon the treatment of different abiotic stresses. Genomic analysis has shown the expansion of the HSP70 family to nine cytosolic members in *P. patens* (Rensing et al., 2008), whereas all algal genomes sequenced to date encode only one single cytosolic HSP70 (van den Wijngaard et al., 2005). The expansion of some of the resistance related genes like *hsp70* during the evolution might help the plant to confront with a more complicated growth environment. Evolutionally, it seems that HSP70s are a crucial part of the plant apparatus ensuring resistance to various abiotic stresses.

SPECIFIC RESPONSES TO EACH TREATMENT

Besides the common responses, *P. patens* also has specific responses to each individual abiotic stress.

DEHYDRATION STRESS

To successfully colonize a terrestrial habitat, the plant should be able to adapt to the environment with an uncertain water supply. Most of the current land plants avoid harmful reductions in internal water supplies by using a variety of anatomical adaptation

Table 1 | Proteins that were commonly regulated by cold (C), salt (S), dehydration (D), and ABA (A) (Wang et al., 2008, 2009a,b, 2010).

Accession no.	Description	C	S	D	A
CAA51071.1	Glyceraldehydes 3-phosphate dehydrogenase	Y	Y	Y	Y
CAB85987.1	dnaK-type molecular chaperone hsc70.1	Y	Y	Y	Y
AAG61085.1	Intracellular pathogenesis-related protein like protein	Y	Y		Y
CAA68141.1	Chloroplast FtsH protease	Y	Y		Y
CAE47464.1	Lipoxygenase	Y	Y		Y
BAC85066.1	ATP synthase α subunit	Y	Y	Y	
NP_904216.1	ATP synthase CF1 α chain	Y	Y	Y	
AAQ88112.1	Actin	Y		Y	Y
BAC85044.1	Rubisco large subunit	Y		Y	Y
AAV65396.1	Physcomitrin		Y	Y	Y
AAY78603.1	PfkB-type carbohydrate kinase family protein		Y	Y	Y
BAC87878.1	Ribulose bisphosphate carboxylase small chain		Y	Y	Y
CAA05547.1	Heat shock protein 70		Y	Y	Y
CAA66484.2	2-Cys peroxiredoxin		Y	Y	Y
CAB75445.1	Fructokinase-like protein		Y	Y	Y
CAB43552.1	Phosphoribosyl diphosphate synthase		Y	Y	Y
CAB79338.1	HSP 70-like protein		Y	Y	Y
CAC43713.1	Unnamed protein product		Y	Y	Y
D84501	Hypothetical protein At2g12170		Y	Y	Y
F96795	Hypothetical protein F28O16.9		Y	Y	Y
Q9LKR3	Luminal binding protein precursor (BiP1) (AtBP1)		Y	Y	Y
BAA83481.1	Rubisco small subunit			Y	Y
BAC85045.1	ATP synthase β subunit		Y		Y
BAD08518.1	Light-harvesting chlorophyll *a*/*b*-binding protein	Y	Y		
BAD08519.1	Light-harvesting chlorophyll *a*/*b*-binding protein 2		Y	Y	
BAD94943.1	AMP deaminase like protein		Y		Y
CAA49506.1	Ketol acid reductoisomerase	Y		Y	
CAA49170.1	Nucleoside diphosphate kinase	Y	Y		
CAA67427.1	Thylakoid-bound ascorbate peroxidase			Y	Y
CAA73616.1	Multicatalytic endopeptidase		Y		Y
CAB87667.1	Subtilisin-like protease-like protein			Y	Y
CAB80488.1	Calcium-dependent protein kinase-like protein			Y	Y
CAB80784.1	AT4g00260		Y		Y
AAB20558.1	Light-regulated glutamine synthetase isoenzyme	Y	Y		
CAB87759.1	14-3-3-like protein GF14 ε	Y	Y		
CAB62598.1	*N*-hydroxycinnamoyl/benzoyltransferase-like protein		Y		Y
CAB54558.1	Plastid division protein ftsZ1			Y	Y
CAB52365.1	ATP synthase gamma chain		Y		
CAC03450	Ser/Thr-specific protein kinase-like protein			Y	Y
CAC42637.1	Unnamed protein product		Y	Y	
CAC43717.1	Unnamed protein product			Y	Y
CAC43712.1	Unnamed protein product			Y	Y
CAD38154.1	Putative ascorbate peroxidase			Y	Y
CAI84534.1	Unnamed protein product		Y		Y

(Continued)

Table 1 | Continued

Accession no.	Description	C	S	D	A
C96608	Hypothetical protein F25p12.91			Y	Y
O65719	Heat shock cognate 70 kDa protein 3		Y		Y
O04005	Peroxiredoxin (thioredoxin peroxidase)			Y	Y
P22954	Heat shock cognate 70 kDa protein 2		Y		Y
Q39102	Heat shock cognate 70 kDa protein 1	Y	Y		
Q43127	Glutamine synthetase	Y	Y		
Q93Z66	Ribose-phosphate pyrophosphokinase 3		Y	Y	
Q9SYM5	Probable rhamnose biosynthetic enzyme 1		Y	Y	
Q39044	Vacuolar processing enzyme, β-isozyme precursor		Y		Y
1RP0A	Chain A, crystal structure of Thil protein		Y	Y	
1RP0B	Chain B, crystal structure of Thil protein		Y	Y	

mechanisms, including root system, vascular tissues, and stomata, cuticles and lignin that restrict evaporative loss of water. However, the less complex plants, including the model moss species – *P. patens*, lack these kinds of anatomical adaptation, which means their primary response to dehydration is at the cellular level. And these cellular responses to water deficit have both economic and evolutionary importance that can affect the agriculture productivity and plant survival.

As mentioned, bryophytes are among the oldest terrestrial land plants. They should have the ability to survive severe dehydration stress. The ability to endure severe water deficit might commonly exist in bryophyte. It is reported that a bryophyte, *Tortula ruralis* is desiccation tolerant (Oliver et al., 2004). Previous studies have shown that the *P. patens* plant could also recover its growth from a 92% water-loss (Frank et al., 2005). But there is still controversy on this point since Koster et al. (2010) reported that it could not survive water potential lower than −13 MPa (corresponding to 91% relative humidity). Pretreatment with ABA can increase *P. patens*' desiccation tolerance and help it to survive a condition of 13% relative humidity (Khandelwal et al., 2010; Koster et al., 2010). Although it lacks the anatomical adaptations that commonly exist in the current dominant terrestrial plants, it could also adjust its cellular structure to avoid the severe damage of the water-loss. Besides the shrink of the cell and the dismantling of chloroplast inner membrane system, the cytoskeleton was also degraded during the drying process. All these features could be supported by the changes of related proteins, such as FtsZ, tubulin, and actin (Wang et al., 2009b). Although the cell shrunk dramatically, the plasma membrane still kept integrated. It seems that the avoidance of plasma membrane damage is very important for plants to survive dehydration. Analysis of the membrane lipid constituents and its changes after the treatment of ABA might be helpful to understand the underlying mechanism.

Previous transcriptome analysis found an actinoporin-like proteins (ALPs) in drought stress (Nishiyama et al., 2003). Recently, Hoang et al. (2009) confirmed the up-regulation of this gene by dehydration and renamed it as bryoporin (*PpBP*). Besides dehydration, ABA, jasmonic acid (JA), salicylic acid (SA), and wound treatments could also up-regulated the expression of this gene. The PpBP protein contains hemolytic activity. Overexpression of this gene could enhance the dehydration tolerance of *P. patens*.

cDNA microarray analyses showed that a bunch of late embryogenesis abundant (LEA) proteins, mainly group 2 and 3, were induced upon the dehydration stress (Oliver et al., 2004; Cuming et al., 2007). This observation is consistent with the data in proteomic analysis. It is well known that the LEA proteins are very important for plant seeds experiencing the desiccation. They were also proved to play critical roles in plant vegetative tissues under drought or other abiotic stresses (Cuming et al., 2007; Hundertmark and Hincha, 2008). Microarray and proteomic analyses indicated that these proteins were mainly induced or up-regulated by dehydration treatment in *P. patens* (Hoang et al., 2009; Wang et al., 2009b). This is also true in *Tortula ruralis* (Oliver et al., 2004). Interestingly, Saavedra et al. (2006) reported that the expression of dehydrin (group 2 LEA protein) encoding gene was up-regulated at both mRNA and protein level by different abiotic stresses including ABA, cold, salt, and dehydration in *P. patens*, which indicates that this protein might be also important in the response to these stresses. However, its function has only been confirmed under dehydration stress. Knockout of this gene could lead to *P. patens* lose the ability to recover from severe osmotic stress (Saavedra et al., 2006). There still need more evidence to show when the expansion of LEA proteins' function from dehydration stress to other abiotic stresses happened during the evolution.

SALT STRESS

The *P. patens* has been shown to be high salt tolerant. The treatment of 300 mM NaCl did not result in any observable differences between the stressed and untreated plants (Oliver et al., 2004; Wang et al., 2008). It could even survive the treatment of NaCl with a concentration up to 350 mM (Oliver et al., 2004).

Compared the differentially displayed proteins under the salt stress with those under other abiotic stresses, we could find that the proteins involved in the modulation of ionic and osmotic homeostasis were specifically regulated by the salt stress. It is well

known that the ion absorption and compartmentalization are crucial for the normal growth of plants. High apoplastic levels of Na^+ and $C1^-$ could alter the aqueous and ionic thermodynamic equilibrium, and hence result in hyperosmotic stress, ionic imbalance, and toxicity. Thus, it is vital for the plant to re-establish its cellular ion homeostasis in high-salinity environments. During exposure to high levels of salinity, the maintenance of K^+ and Na^+ homeostasis is crucial, which depends on the proton-motive force created by the action of H^+-ATPases and H^+-pyrophosphatases (Hasegawa et al., 2000). Phototropin and the 14-3-3 protein are primarily function as signaling pathway components (Wu et al., 1997; Takemiya et al., 2005). Recently, researchers have shown that these two proteins could work either cooperatively or independently to regulate the function of plasma membrane H^+-ATPases and hence control the opening of stomatal and ion channels (e.g., the K^+ channel; Inoue et al., 2005; van den Wijngaard et al., 2005). A PIIB-type Ca^{2+}-ATPase, which involved in salt induced Ca^{2+} signaling is also essential for *P. patens*' salt tolerance (Qudeimat et al., 2008). In addition to these proteins, ABC transporters may also involved in the regulation of homeostasis, as it does in yeast (Miyahara et al., 1996). Chloride can interfere with anionic sites involved in the binding of RNA and sugar-phosphates (Serrano, 1996; Hasegawa et al., 2000). Down-regulation of the chloride channel protein limits the transport of $C1^-$ into cell. *P. patens* might be able to increase its salinity tolerance through constraining the transport of Na^+ and Cl^-. This phenomenon has also been reported in tomato (Estan et al., 2005). Comparative analysis of the microarray data between *P. patens* and *Arabidopsis* showed that the functions of some other proteins such as DREB-like, Dof, and bHLH TAPs in salt response might be conserved during evolution (Richardt et al., 2010).

COLD STRESS

Previously, it has been shown that the *P. patens* could survive the treatment of −4°C (Sun et al., 2007). The authors also showed that 0°C treatment could induce the *P. patens* to be resistant to a temperature as low as −7°C. These results indicate that 0°C treatment could initiate some biochemical or physiological processes that help *P. patens* to be more resistant to the freezing temperature. It is reported that pretreatment with ABA, NaCl and mannitol could increase the freezing tolerance of *P. patens* (Minami et al., 2003). In spite of this, the response of *P. patens* to cold treatment has little similarity with that to desiccation and salinity as we have mentioned above. One prominent characteristic of the cold response is that the expression of a series of transcription factors changed upon the treatment. The number of transcription factors is much more than those in any other treatment (Wang et al., 2009a). It seems that there are more regulation happened at the transcription level in response to the cold stress. Quantitative real time (RT)-PCR data for some of the selected genes showed that the changes at the protein and RNA level are not always consistent.

ABA TREATMENT

Plant hormones are important in regulating plant growth and development and its response to a variety of biotic and abiotic stresses. Significant progress has been made in identifying the key components involved in the signaling pathway of different plant hormones. Based on the current knowledge, ABA is known to play a crucial role in cellular responses to environmental stresses such as drought, cold, salt, wounding, UV radiation, and pathogen attack (Rock, 2000). The ABA signaling pathway was found in *P. patens* (Knight et al., 1995), and some key components in this pathway, such as ABI3, ABI1 were also characterized (Marella et al., 2006; Komatsu et al., 2009). The study in *P. patens* will be useful to understand the evolution of ABA signaling pathway.

The signaling pathways of dehydration and salinity were regarded as ABA dependent, which might help to explain the result that most of the proteins regulated by ABA were also regulated by the salinity and dehydration (Wang et al., 2008, 2009b, 2010). Unexpectedly, microarray analysis showed that very little genes were commonly regulated by salt, dehydration, and ABA (Saavedra et al., 2006). This implies that a systematic proteomic and transcriptomic analysis is necessary. In spite of this, the pattern of the protein changes in the ABA treatment is distinct from those in the dehydration and salt treatments (Wang et al., 2008, 2009b, 2010). Proteomic analysis data showed that ABA treatment could regulate more defense related proteins and transcription factors than salinity and desiccation could (**Figure 2**). Specifically, some defense related proteins and transcription factors, such as receptor-like kinase, disease resistance proteins, lipoxygenase, and WRKY transcription factor 52, were exclusively up-regulated by ABA in our proteomic studies (Wang et al., 2010). This may be explained by the fact that ABA involved in not only the dehydration and salinity signaling pathway but also the response to some other stresses. In addition to these proteins, we also found that some cell growth related proteins, such as expansin and extensin-like protein, were only regulated by ABA in all of the treatments. Expansin was known to have cell wall loosening activity and to be involved in the cell expansion and enlargement (Cosgrove, 2000). Previously, it has been shown that expansin is linked to the action of auxin (McQueen-Mason et al., 1992), gibberellin (Cho and Kende, 1997), cytokinin (Downes and Crowell, 1998), ethylene (Cho and Cosgrove, 2002), and brassinosteroids (Sun et al., 2005). Our proteomic data (Wang et al., 2010) indicate that the expansin is also related to the function of plant hormone ABA at least in the very early terrestrial plant species. Prediction of the ABA responsive genes was conducted based on the *cis*-regulatory elements (Timmerhaus et al., 2011), and over 90% of the predicted target genes were validated by microarray analysis (Richardt et al., 2010). Taking these data together, we conclude that the ABA signaling pathway was largely conserved since the first land plants during the evolution.

CONCLUSION

Physcomitrella patens has been a model system for decades because of its advantages in both genetic and structural aspects. Besides, *P. patens* has been proved to be highly resistant to different extreme abiotic stresses, such as high salinity, severe dehydration, and freezing temperature. With the availability of its genome information in the public database, exploring the stress resistance mechanism of this moss species at the proteomic level comes into reality. We have conducted a systematic proteomic analysis about its response to different abiotic stresses including high salinity, severe dehydration, cold, and ABA treatments. Except for the distinct responses

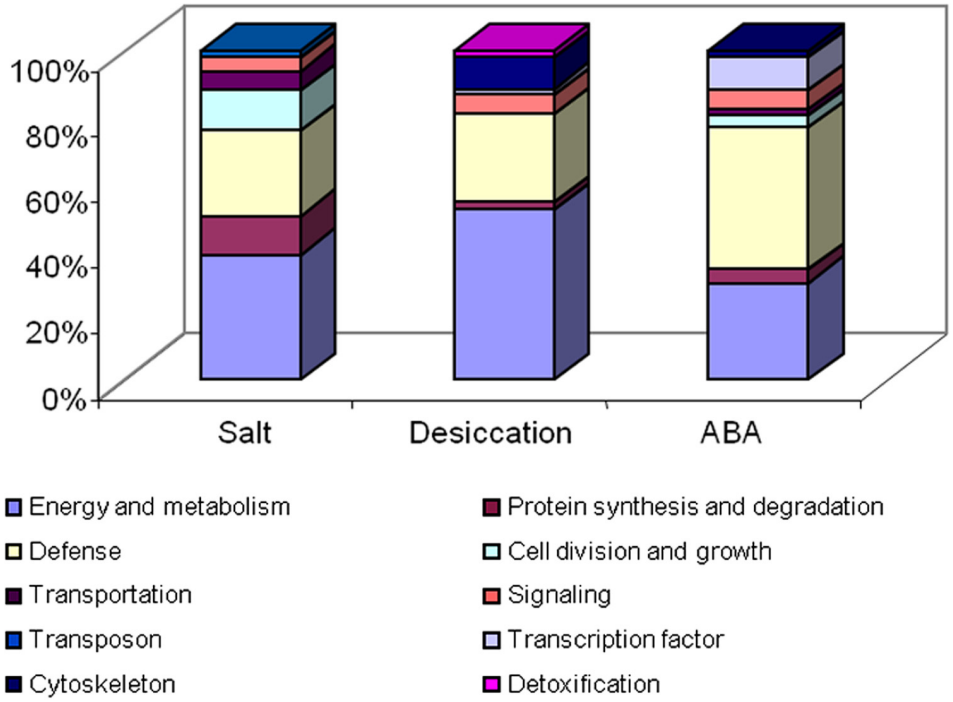

FIGURE 2 | Functional categorization of the differential displayed proteins under the treatment of salt, desiccation, and ABA. This figure was based on our previous data (Wang et al., 2008, 2009a,b, 2010).

to each treatment, common responses also exist. Comparison the responses between different treatments implies that there is a closer relationship between ABA and salt or ABA and dehydration than that between ABA and cold. Further functional analysis for those differentially displayed proteins under different stresses will help us to get more understanding to the stress resistance mechanisms in *P. patens* or even the whole plant kingdom.

As mentioned above, proteomic analyses have shown that a series of proteins involved in various cellular functions were regulated by different abiotic stress. To obtain further information about how these processes respond to stresses and how gene expressions were regulated, it might be necessary to initiate the sub-proteomic study in *P. patens*. To our understanding, this includes three major parts. First, it is about the proteomic analysis of organelles, such as nucleus, chloroplast, and mitochondria. Secondly, since the abiotic could result in ROS stress in the cell, and the accumulation of ROS in cell thereafter could lead to the carbonylation of the proteins, analysis of the protein carbonylation might be helpful to understand the protein turnover and functional alteration in response to stress. Thirdly, stress responses start with signal transduction. Currently, the knowledge of signaling in *P. patens* is still absent. Phosphoproteomics study might contribute a lot for us to understand the signaling pathway involved in stress responses. Furthermore, comparing the data acquired in *P. patens* with those from other model plants such as *Arabidopsis* and rice will bring us more general and comprehensive knowledge about the mechanisms of abiotic stress response in the plant kingdom.

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Proteomics of rice grain under high temperature stress

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Recent proteomic analyses revealed dynamic changes of metabolisms during rice grain development. Interestingly, proteins involved in glycolysis, citric acid cycle, lipid metabolism, and proteolysis were accumulated at higher levels in mature grain than those of developing stages. High temperature (HT) stress in rice ripening period causes damaged (chalky) grains which have loosely packed round shape starch granules. The HT stress response on protein expression is complicated, and the molecular mechanism of the chalking of grain is obscure yet. Here, the current state on the proteomics research of rice grain grown under HT stress is briefly overviewed.

Keywords: α-amylase, chalky grain, high temperature stress, *Oryza sativa*, ripening, starch

INTRODUCTION

The Intergovernmental Panel on Climate Change is discussing several scenarios concerning the greenhouse gas emission, and it is predicted that the global surface temperature will further increase during the twenty-first century. High temperature (HT) impediment in developing stage of crops, such as rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum* spp.), barley (*Hordeum vulgare*) and soybean (*Glycine max*), has been occurred due to the impact of global warming (Ainsworth and Ort, 2010). Rice production is known to be sensitive to increasing environmental temperature (Peng et al., 2004), and current grain filling temperatures are already approaching critical levels in many countries with rice cultivation (Ainsworth, 2008). Furthermore, it should be stressed that the grain quality is more susceptible to the HT stress compared with the grain yield. The appearance quality of rice grain is mainly evaluated by its transparency. As shown in **Figure 1**, the perfect grain is filled with normal starch granules exhibited polygonal with sharp edges. In the case of damaged (central chalky) grains caused by the HT stress, abnormal and round shape starch granules were loosely packed in the part of grain, and this part is whitely seen by irregular reflection of the light. The mechanism of grain chalkiness under HT stress is considerably complicated. The temperature at the grain filling stage has shown to influence the starch composition in rice grains (Asaoka et al., 1984, 1985, 1989; Inouchi et al., 2000; Lisle et al., 2000; Umemoto and Terashima, 2002; Cheng et al., 2005; Yamakawa et al., 2007). Heat stress reduced the amylose contents and weakly changed the fine structure of amylopectin (Asaoka et al., 1984; Inouchi et al., 2000), possibly indicating that the abnormal expression of the starch synthesizing enzymes is a key factor causing the chalky grains of rice (Nishi et al., 2001; Tanaka et al., 2004). However, chalky grains without any remarkable change in starch chain distribution were also observed compared to translucent grains which were ripened under both control and HT (control + 3.6°C) conditions (Tsutsui et al., 2013).

Recent remarkable development of the efficient, sensitive, and high-throughput proteomics technology lead us to the next research phase of the grain filling. In the mini review, the current progress of studies on proteome of rice ripening and mature grains is described. Furthermore, the chalking mechanism of rice grain under the HT stress is discussed in terms of grain starch glycome, transcriptome, and proteome.

RICE GRAIN PROTEOME UNDER HT STRESS

Comprehensive proteomic survey of metabolic enzymes, structural and storage proteins, and allergens in rice grains have been carried out using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and gel-free-based shotgun technologies (Koller et al., 2002; Lin et al., 2005; Xu et al., 2008; Lee and Koh, 2011). Lee and Koh (2011) demonstrated that the identification of 4,172 non-redundant proteins with a wide range of molecular weight (5.2–611 kDa) and pI values (pH 2.9–12.6) in developing and mature grains of rice. In the analysis of ontology category enrichment for the 4,172 proteins, 52 categories were enriched, including the carbohydrate metabolic process, transport, localization, lipid metabolic process, and secondary metabolic process. Expression analyses of protein groups associated with different functional categories revealed dynamic changes of metabolisms

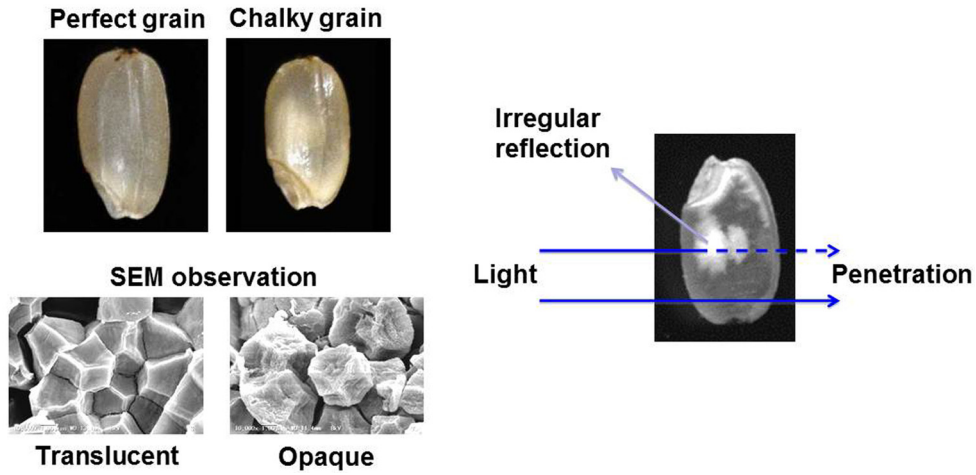

FIGURE 1 | Perfect and chalky grains of rice. Bars: 3.33 μm.

during rice grain development. It seems that a switch from central carbon metabolism to alcohol fermentation is important for starch synthesis and accumulation in the development process (Xu et al., 2008). Interestingly, however, it was detected that proteins involved in glycolysis, citric acid cycle, lipid metabolism, and proteolysis, and so on, accumulated at higher levels in mature grain than those of developing stages (Lee and Koh, 2011). This observation appears to indicate that the preparation of materials required in germination occurred until the seeds were fully matured and dried.

Information of rice grain proteome in the anthesis and ripening stages under HT stress was limited. The anthesis is the most sensitive stage to HT stress in rice. Jagadish et al. (2010) performed gel-based proteomic analyses of different genotype anthers prepared from rice plants exposed to 6 h of high (38°C) and control (29°C) temperature at anthesis. Both cold (19 kDa) and heat (24 kDa) shock proteins were found significantly up-regulated in highly heat tolerant genotype N22, suggesting that these might contribute to the greater heat tolerance of N22. Lin et al. (2005) have reported that HT stress (35/30°C) during caryopsis development reduced the expression of starch granule-bound starch synthase (Wx), allergen-like proteins, and elongation factor 1β, but enhanced the expression of small heat shock proteins (sHSP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and prolamin, in comparison with those in control temperature (30/25°C). Furthermore, they analyzed HT stress response of several different cultivars including high-chalky types, the results showing that sHSP was positively correlated with the appearance of chalky kernels (Lin et al., 2005). Transgenic rice plants overexpressing HSP17.7 (Murakami et al., 2004) and HSFA4d (Yamanouchi et al., 2002) exhibited increase of heat tolerance, however, there was no evaluation during grain filling stage. In recent studies, the accumulation of all classes of storage proteins was increased at early ripening stage under the HT stress, whereas the prolamin accumulation was decreased at maturation and desiccation stages (Lin et al., 2010). On the other hand, Li et al. (2011) descried that pyruvate phosphate dikinase (PPDK) was up-regulated and pullulanase (PUL) was down-regulated during grain filling under the grain chalkiness induced temperature condition, respectively. In wheat grain proteome, HSPs, storage proteins, late embryogenesis abundant proteins, peroxiredoxins, and α-amylase/trypsin inhibitors have shown to be HT-responsive (Skylas et al., 2002; Majoul et al., 2003; Hurkman et al., 2009; Yang et al., 2011). Thus, it must be said that the information concerning the protein expression in developing seeds response to the HT stress is minimum and confusing.

STARCH GLYCOMIC, TRANSCRIPTOMIC, AND PROTEOMIC ASPECTS OF GRAIN CHALKINESS

Grain chalking caused by HT stress during ripening stage is one of the major issues decreasing the appearance quality of rice grain (Yoshida and Hara, 1977; Tashiro and Wardlaw, 1991a,b). Although the chalky grains of rice were appeared even at the optimum temperature range of grain filling, the HT stress increased the percentage of chalky grains and further extended the chalking area of grain (Tsutsui et al., 2013). Scanning electron microscopy (SEM) studies of the chalky grains have been done by several research groups (Evers and Juliano, 1976; Tashiro and Wardlaw, 1991a; Kim et al., 2000; Lisle et al., 2000; Zakaria et al., 2002). The starch granules in translucent part of chalky grain had similar tight packing and shape to the perfect grain. While, in the opaque part of chalky grains, the starch granules that had a round shape with several small pits were loosely packed (Tsutsui et al., 2013).

It has been demonstrated that the environmental temperature at the ripening stage apparently changes the starch composition in rice grains (Asaoka et al., 1984; Inouchi et al., 2000; Lisle et al., 2000; Umemoto and Terashima, 2002; Cheng et al., 2005; Yamakawa et al., 2007). Interestingly, the enzyme activity of starch branching enzyme IIb (BEIIb) *in vitro* was shown to drop sharply at more than 35°C (Ohdan et al., 2011). The HT stress decreased the amylose contents and the weight ratio of A + short B chains to long B chains of amylopectin in grain (Asaoka et al., 1984; Inouchi et al., 2000), while opposite directions of changes in A- and B-fractions were observed at lower temperatures (Umemoto et al., 1999). Microarray analysis of rice ripening

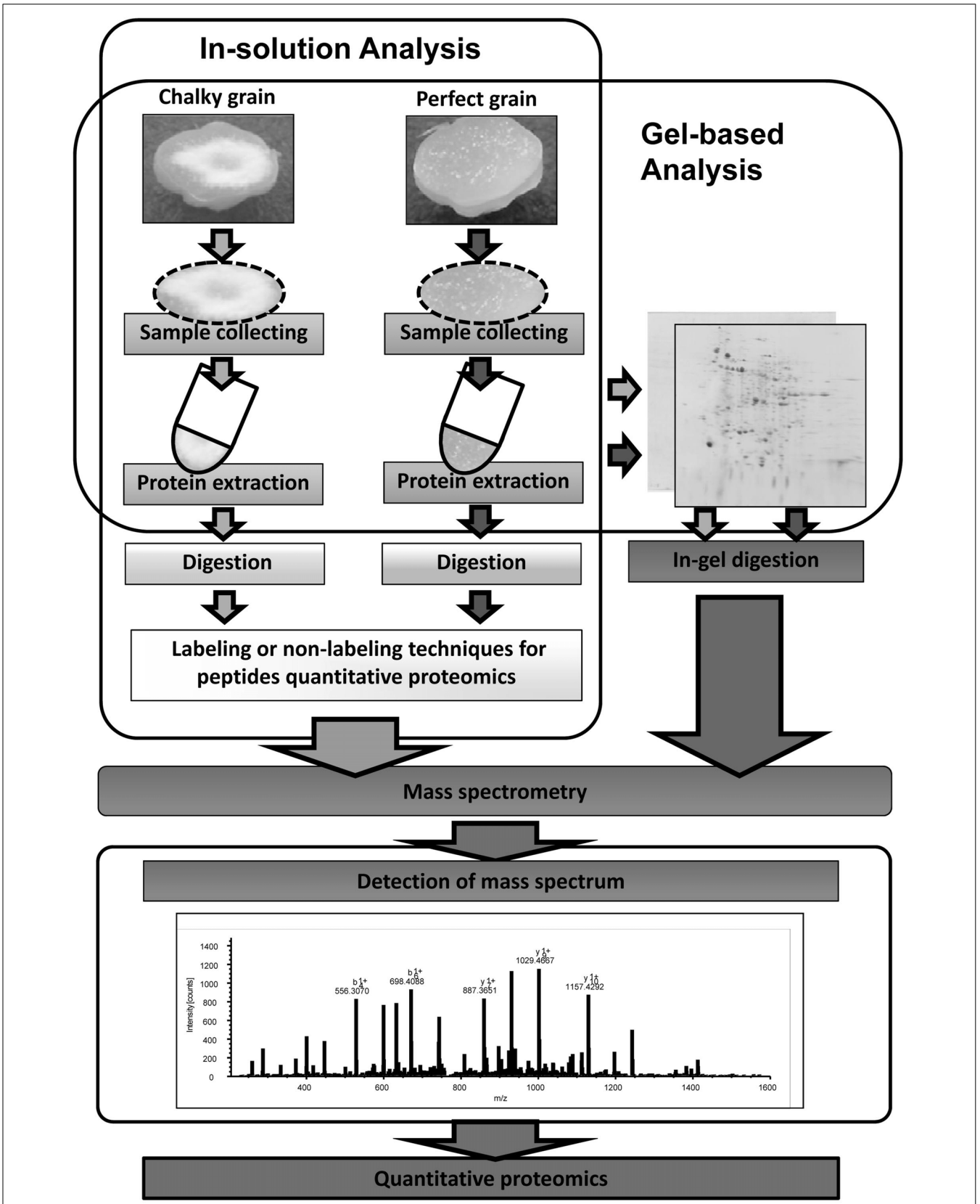

FIGURE 2 | Local proteomic strategies for understanding the chalking mechanisms of rice grains.

seeds showed that the expression of starch synthesis-related genes including granule-bound starch synthase I (*GBSSI*), *BEIIb*, ADP-glucose pyrophosphorylase (*AGPS2b, AGPS1, and AGPL2*) and ADP-glucose translocator (*BT1-2*) were partially repressed under HT condition (Yamakawa et al., 2007). It was widely accepted that the *amylose-extender* (*ae*) mutant, that is deficient in *BEIIb* gene, exhibited a severe chalky phenotype of grain. The *ae*-type grains contained amylopectin with largely reduced amount of short chains of degrees of polymerization (DP) 8–12 and enriched in long chains with DP more than 19 (Nishi et al., 2001). The grain chalkiness of *ae* mutant was disappeared by transforming the wild-type *BEIIb* gene (Tanaka et al., 2004), suggesting that the abnormal expression of *BEIIb* is one of factors causing the chalking of grain. However, a recent study showed that the chain-length distributions of starches prepared from the translucent and opaque parts of perfect and chalky grains of Koshihikari cultivar harvested in 2009 (24.4°C) and 2010 (28.0°C) were not distinguishable (Tsutsui et al., 2013). Moreover, Yamakawa et al. (2007) have described that the reduction of amylose content and the increase of long B chains of amylopectin by HT were not correlated to the grain chalkiness. Thus, the relevance of the starch fine structure to the chalkiness of grain under HT stress is still unclear.

The SEM observation indicated that the opaque portion of chalky grain had looser packing of round-shaped starch granules, furthermore, numerous pits were observed on the surface of starch granule from the chalky endosperm (Tashiro and Wardlaw, 1991a; Tsutsui et al., 2013). These observations suggested that, in addition to damage of starch synthesis, premature autolysis of starch induced by HT stress of ripening stage resulted in the abnormal shape of starch granules in the opaque parts of grain. Recently, it was observed that the level of glucose in opaque parts was strikingly high in comparison with the corresponding translucent parts of perfect grains (Tsutsui et al., 2013), possibly indicating that amylolytic enzymes exist and work in the opaque parts of chalky grains. The expression of several α-amylase mRNA species was detected in ripening seeds of rice using a transcriptomic analysis. Noteworthy, the mRNA expression of *Amy1A*, *Amy1C*, *Amy3D*, and *Amy3E* genes, as well as α-amylase activity, was increased under HT stress (Yamakawa et al., 2007; Hakata et al., 2012). In addition, cauliflower mosaic virus 35S promoter-driven overexpression of AmyI-1 (*Amy1A*) and AmyII-4 (*Amy3D*) resulted in grains with decrease weight and chalky appearance even under normal temperature condition (Asatsuma et al., 2006). In marked contrast to the above story, Ishimaru et al. (2009) reported that the α-amylase mRNA was not detected in the central part of endosperm tissue during grain filling, claiming that starch degradation by α-amylase was not the cause of the formation of chalky grain. However, Hakata et al. (2012) have shown that RNAi-mediated suppression of α-amylase genes in ripening seeds resulted in fewer chalky grains under HT conditions, and the extent of the decrease in the ratio of chalky grains was highly correlated to decreases in the gene expression of *Amy1A*, *Amy1C*, *Amy3A*, and *Amy3B*. Furthermore, Tsuyukubo et al. (2010, 2012) have demonstrated by immunoblotting with the specific antibodies that AmyI-1 (Amy1A) and AmyII-4 (Amy3D) proteins existed in the outer layers (100–80% fractions) of rice grain (cv. Koshihikari), while α-glucosidase and AmyII-3 (Amy3E) were mainly detected in the inner layers (90–0% fractions). These experimental results would reveal that activation of amylolytic enzymes by HT is a crucial trigger for grain chalkiness. Local proteomic analyses for determining individual contribution of starch degrading enzymes involving in site-specific localization of chalking remain to be performed.

FUTURE PERSPECTIVE

Global warming is the most serious environmental issue, and the global surface temperature will probably rise a further 1–6°C during the twenty-first century. HT stress in rice ripening periods causes a decrease in not only grain yield but also grain quality. Grain chalking caused by HT stress during ripening stage is one of the major problems in the field of agriculture. Understanding of the mechanisms of grain chalking under HT stress in ripening is extremely important to develop a strategy for reducing the large occurrence of chalky grains in the region to produce good taste and high quality of rice by climate warming. Intensive and precise local proteomic analyses (see **Figure 2**) of HT-stressed developing and mature grains will gain better understanding the grain chalking mechanism(s).

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Plant plasma membrane proteomics for improving cold tolerance

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Plants are always exposed to various stresses. We have focused on freezing stress, which causes serious problems for agricultural management. When plants suffer freeze-induced damage, the plasma membrane is thought to be the primary site of injury because of its central role in regulation of various cellular processes. Cold tolerant species, however, adapt to such freezing conditions by modifying cellular components and functions (cold acclimation). One of the most important adaptation mechanisms to freezing is alteration of plasma membrane compositions and functions. Advanced proteomic technologies have succeeded in identification of many candidates that may play roles in adaptation of the plasma membrane to freezing stress. Proteomics results suggest that adaptations of plasma membrane functions to low temperature are associated with alterations of protein compositions during cold acclimation. Some of proteins identified by proteomic approaches have been verified their functional roles in freezing tolerance mechanisms further. Thus, accumulation of proteomic results in the plasma membrane is of importance for application to molecular breeding efforts to increase cold tolerance in crops.

Keywords: plasma membrane, cold acclimation, freezing tolerance, abiotic stress, shotgun proteomics, gel-based proteomics

INTRODUCTION

Survival of plants is considerably dependent on their ability to adapt to environmental conditions under which they live. Because of their immobility, plants need to recognize and respond to external stimuli sensitively and properly for their survival. Without proper signal perception and subsequent responses, they can be killed or injured rapidly and instantaneously. External stimuli to influence plant's life include a number of different factors resulting from biotic stresses (e.g., microorganism infection and wounding by herbivores) and abiotic stresses (e.g., drought, salt, flooding, light, nutrition, physical pressure, and extreme temperatures).

The plasma membrane, one of the semi-permeable cellular membranes, is the membrane that separates the intracellular and extracellular spaces, and plays an important role in the exchange of compounds such as metal ions, metabolites, and nutrients. Signals from extracellular stimuli may also be transmitted through the plasma membrane, most of which are likely conducted by plasma membrane proteins. In addition, the plasma membrane itself needs to withstand stresses from the extracellular space. Therefore, plant cells that perceive stress stimuli are thought to alter the proteomic properties of the plasma membrane to adapt to stresses. Management of the effects of these stress-related stimuli is important for improvement of growth performance, yield and quality of crops in the field. For example, crops growing in most American farmlands potentially face threats from drought, heat, and cold stresses, and it was estimated that drought and heat stresses caused damage of US$4.2 billion in August 2000 (Mittler, 2006).

Many plants that live in temperate regions, including important crop species such as wheat, rye, and barley, increase their freezing tolerance when air temperature decreases and day length shortens, which is called cold acclimation (Levitt, 1980). In particular, alterations of plasma membrane proteins during cold acclimation have been well characterized using biochemical and physiological approaches and recognized as a critical adaptation mechanism to low temperature (Steponkus, 1984; Uemura and Yoshida, 1984; Uemura et al., 2006). More specifically, during cold acclimation, increased P-type ATPase activity, disassembly of microtubules and accumulation of several dehydrin family proteins occur on the plasma membrane (Ishikawa and Yoshida, 1985; Abdrakhamanova et al., 2003; Kosová et al., 2007). These changes were also confirmed by semi-proteomic analyses (Kawamura and Uemura, 2003). However, these studies were focused on specific proteins or at most on proteins that were identified on two-dimensional electrophoresis (2-DE) gels as cold-responsive proteins and not performed in a large scale to obtain comprehensive data sets of the candidate proteins in the plasma membrane. Proteomics of the plasma membrane with a help of rapid advance of analytical techniques using mass spectrometry will provide us information that suggests us a number of associations of the plasma membrane proteins and cold acclimation mechanism in a relatively short period. Thus, elucidation of proteome profiles of the plasma membrane when exposed to low temperature in combination with genetic and physiological studies will be valuable for practical improvement of plant cold tolerance.

RESPONSES OF PLASMA MEMBRANE PROTEOME TO ABIOTIC STRESSES

In this section, we review studies of proteomic changes of the plasma membrane in association with abiotic stresses. Abiotic stresses (e.g., drought, salt, flooding, light, nutrition, physical pressure, and extreme temperatures) generally restrict plant distribution, growth and reproduction, and sometimes result in severe damage to plants. Plasma membrane proteomics have revealed the possibility that plants have adaptation systems against abiotic stress factors in association with plasma membrane functions. For example, the relationship between plasma membrane and salt stress has been extensively investigated. Nohzadeh et al. (2007) and Cheng et al. (2009) performed comprehensive proteome analysis of rice root plasma membranes based on 2-DE mapping. Nohzadeh et al. (2007) identified eight proteins that responded to salt stress. These proteins included 14-3-3 proteins, which are well known as proton-ATPase regulators (Babakov et al., 2000) and may be involved in pH regulation in cells under salt stress conditions. Cheng et al. (2009) also identified 18 salt-responsive proteins. Proteomic analyses combined with immuno-histochemical analysis of the proteins revealed that a leucine-rich-repeat type receptor-like protein kinase, OsRPK1, which responded to salt stress, accumulated in the cortex plasma membrane under salt stress conditions. Nouri and Komatsu (2010) and Komatsu et al. (2009) analyzed osmotic and flooding stress responses, respectively, of the soybean plasma membrane proteome. They identified dozens of stress-responsive plasma membrane proteins using 2-DE- and nano-LC-MS/MS based proteomics approaches. These results suggest that the plasma membrane has functions that are important for adaptation to osmotic and flooding stresses via ion homeostasis and antioxidative mechanisms.

PLASMA MEMBRANE PROTEOMIC APPLICATIONS FOR INCREASING COLD TOLERANCE

Temperature is one of the major regulatory factors of crop production. In particular, freezing temperatures are accompanied by a state change from water to ice in plants, which has dramatic effects on cellular metabolism. When plants are placed under low temperature conditions, the plasma membrane will also fall into functional decline (i.e., decline of membrane fluidity, transport and metabolic activity and ion homeostasis maintenance, disturbance of signaling processes, and freeze-induced fusion with other intracellular membranes because of physical pressure by extracellular ice formation). Plant plasma membrane, however, has the ability to adapt to freezing temperatures through cold acclimation processes and the plasma membrane proteome is thought to be tightly associated with determination of plant freezing tolerance (**Figure 1**). Compositional changes of plasma membrane proteins and lipids have been well described (Uemura and Yoshida, 1984; Uemura and Steponkus, 1994; Webb et al., 1994; Uemura et al., 1995). On the protein side, Kawamura and Uemura (2003) first applied mass spectrometric technology to elucidate the relationship between plasma membrane proteins and cold acclimation and successfully identified 38 proteins the levels of which changed during 3 days of cold acclimation treatment in *Arabidopsis*. These cold-responsive plasma membrane proteins include early responsive to dehydration proteins (ERD10 and ERD14), which are members of the dehydrins and may protect proteins and membranes against freeze-induced dehydration (Uemura et al., 2006; Kosová et al., 2007). In addition, a novel cold acclimation-induced protein, plant synaptotagmin 1 (SYT1), was identified. In animal cells, an isoform of the synaptotagmin family is known to be associated with membrane resealing (Reddy et al., 2001). Thus, they hypothesized that SYT1 is involved in repairing the plasma membrane when it is disrupted during the freeze-thaw cycle. Yamazaki et al. (2008) later confirmed that synaptotagmin-associated membrane resealing is eventually involved in plant freezing tolerance mechanism. Li et al. (2012) recently applied shotgun proteomics technology to show proteome profiles of the plasma membrane during cold acclimation or abscisic acid (ABA) treatment in *Arabidopsis* suspension cultured cells. ABA is well known to play a key role in plant cold acclimation (Chen and Gusta, 1983; Bakht et al., 2006). Their data suggested that a subset of cold acclimation-induced changes of plasma membrane proteins were mimicked by ABA treatment, but another set of proteins was characteristically changed only by cold acclimation treatment and not by ABA.

Furthermore, Minami et al. (2009) performed proteome profiling to characterize alterations of the *Arabidopsis* microdomain during cold acclimation. The microdomain is a lateral membrane subdomain composed of specific lipids and proteins in the plasma membrane (Simons and Ikonen, 1997). Microdomain proteomics offered new insight into changes and functions of the plasma membrane proteome during cold acclimation. Many functional proteins that are associated with cold acclimation, such as P-type ATPases, aquaporins, and tubulins (Ishikawa and Yoshida, 1985; Abdrakhamanova et al., 2003; Peng et al., 2008), accumulated in microdomain fractions. Minami et al. (2009) found that clathrins and dynamin-related proteins, which are associated with the clathrin-dependent endocytosis pathway, increased in the microdomain during cold acclimation. They predicted that functional changes of endocytosis activity during cold acclimation are regulated by microdomain-enriched clathrins and dynamin-related proteins. Recently, we reported plasma membrane microdomain proteomes isolated from oat and rye (Takahashi et al., 2012). Although oat and rye are monocotyledonous plants and *Arabidopsis* is a dicotyledonous plant, the microdomain proteome profiles of these three species were quite similar (Borner et al., 2005; Minami et al., 2009; Takahashi et al., 2012). Interestingly, oat and rye are phylogenetically related but have quite different freezing tolerance after cold acclimation (Webb et al., 1994). Now, we are conducting analysis to determine the relationship between alterations of the microdomain proteome during cold acclimation and freezing tolerance mechanism. Because monocotyledonous plants contain a number of important crops, such as rice and wheat as well as oat and rye, plasma membrane proteomics in monocotyledonous plants is important for practical improvement of crop freezing tolerance. Along with this line of research, we have just initiated a new project using shotgun proteomics to analyze the plasma membrane proteome during cold acclimation using a novel model monocotyledonous plant, *Brachypodium distachyon* (Draper et al., 2001; The International Brachypodium Initiative, 2010). Information from plasma membrane proteomes during cold acclimation in *B. distachyon* will contribute to

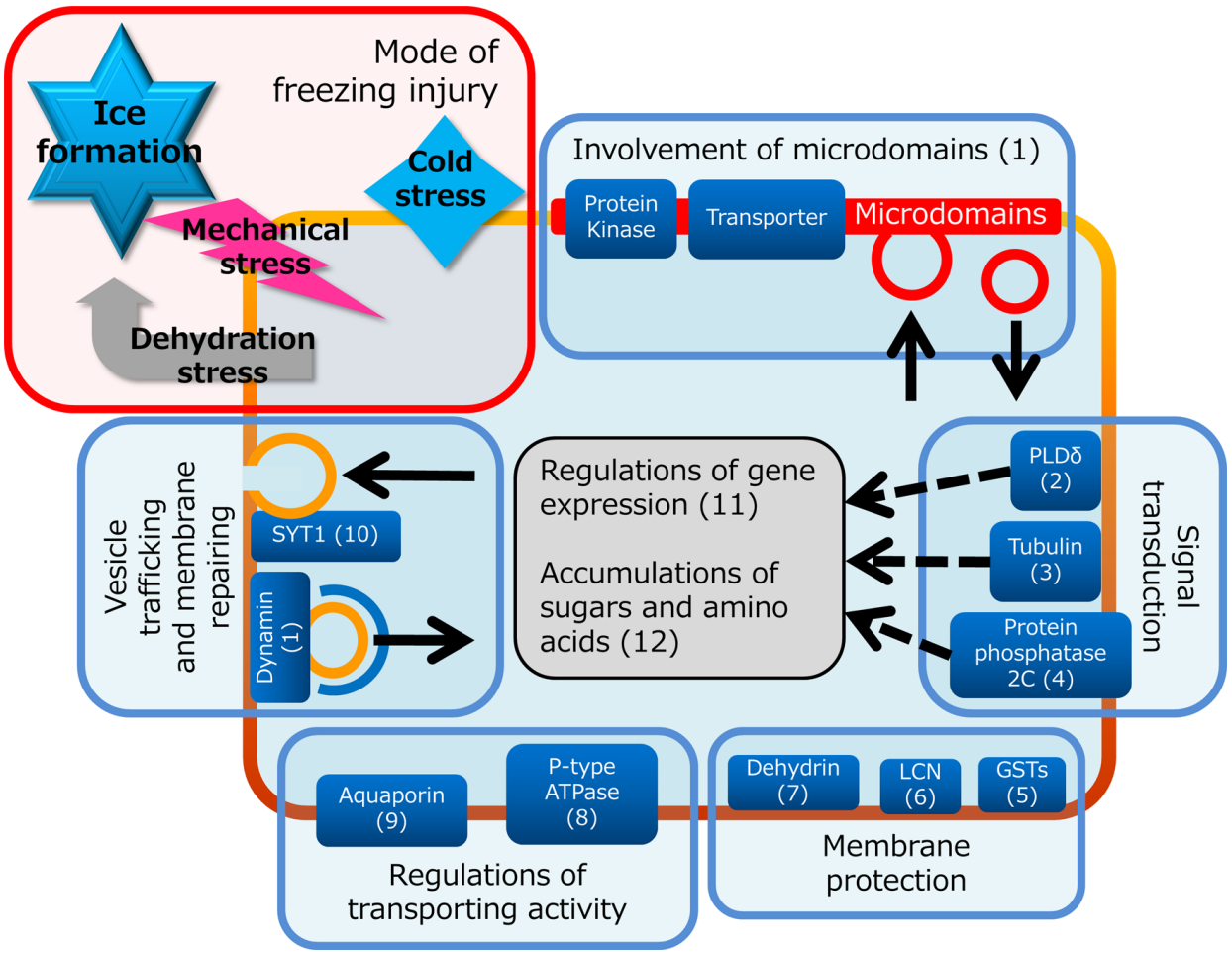

FIGURE 1 | Representative functions of the plasma membrane during cold acclimation suggested by previous proteomics studies. Freezing temperature causes different types of injury including membrane destabilization and protein inactivation resulted from two primary stresses, dehydration stress and mechanical stress, imposed by ice crystals formed in extracellular space. Plant cells can respond to these stresses through signal transduction and gene regulation pathway, and counter these stresses with cold-evoked membrane protection system, optimization of transporting activity and adjustment of vesicle trafficking and membrane patch-up system. Each mechanism related to response to freezing has possibilities to be mediated by proteins and/or lipid environments in microdomains that consist of specific proteins and lipids. Detailed information of each part is referred to following papers: (1) Minami et al., 2009; (2) Li et al., 2004; (3) Abdrakhamanova et al., 2003; (4) Tähtiharju and Palva, 2001; (5) Seppänena et al., 2000; (6) Uemura et al., 2006; (7) Danyluk et al., 1998; (8) Ishikawa and Yoshida, 1985; (9) Peng et al., 2008; (10) Yamazaki et al., 2008; (11) Chinnusamy et al., 2007; (12) Koster and Lynch, 1992.

the improvement of freezing tolerance in agriculturally important crops and increase the cultivated land available for these crops.

SIGNIFICANCE OF THE PLASMA MEMBRANE PROTEOMICS IN APPLICATION TO BREEDING COLD-TOLERANT CROPS

As described above, the plasma membrane plays significant roles in signal perception and cellular homeostasis, and plasma membrane proteins are the most important factors in determining the environmental stress tolerance of plants. Thus, plasma membrane proteomics helps to understand how plants adapt to stressful conditions and to consider how we improve crop production in severe environments. In terms of plant freezing tolerance, expression of several plasma membrane proteins has been genetically modified, which resulted in an increase in freezing tolerance. For example, phospholipase Dδ (PLDδ), a plasma membrane-associated protein which hydrolyzes membrane phospholipids and generates phosphatidic acid (PA), has been confirmed to increase in its amount during cold acclimation by a proteomic approach using *Arabidopsis* (Kawamura and Uemura, 2003). Knock-out of *PLD*δ gene resulted in decreased freezing tolerance, and further, overexpression resulted in increased freezing tolerance when compared with that of wild-type plants (Li et al., 2004). After freezing, higher levels of PA were observed in *PLD*δ-overexpressed plants. Because PA has been thought to be a signal molecule (Bargmann and Munnik, 2006), PA-mediated cellular functions may have critical roles in enhancement of freezing tolerance. Lipocalin-like protein (temperature-induced lipocalin, TIL), another plasma membrane protein that responds to cold treatment (Kawamura and Uemura, 2003), has a positive effect in freezing tolerance. Overexpression

of TIL resulted in higher survival rates at freezing temperature (Tominaga et al., 2006; Uemura et al., 2006). However, molecular mechanism of the effect of LCN on freezing tolerance is still to be determined. Information obtained from proteomic results has plowed ahead with findings of novel factors in association with cold tolerance and contributed to improvement of tolerance to low temperature under laboratory conditions. Now we are at the stage that the proteomic information and the knowledge of cold adaptation and freezing tolerance mechanisms can be combined and with helps of different approaches such as genetics and molecular biology, we can go further to organize collaborative research for application of the proteome information to conventional bleeding or genetic engineering of crops with candidate proteins revealed by proteomic approaches.

FUTURE PERSPECTIVES

Much information about cold tolerance and the plasma membrane proteome is becoming available for the breeding of crops that are productive in cold conditions. Because the molecular mechanisms of freezing stress tolerance are known to share commonalities with tolerance to other abiotic stresses such as drought and salt stress (Mahajan and Tuteja, 2005), the knowledge of cold-responsive plasma membrane proteins may be useful for producing crops tolerant to multiple abiotic stresses.

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Crop and medicinal plants proteomics in response to salt stress

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Increasing of world population marks a serious need to create new crop cultivars and medicinal plants with high growth and production at any environmental situations. Among the environmental unfavorable conditions, salinity is the most widespread in the world. Crop production and growth severely decreases under salt stress; however, some crop cultivars show significant tolerance against the negative effects of salinity. Among salt stress responses of crops, proteomic responses play a pivotal role in their ability to cope with it and have become the main center of notification. Many physiological responses are detectable in terms of protein increase and decrease even before physiological responses take place. Thus proteomic approach makes a short cut in the way of inferring how crops response to salt stress. Nowadays many salt-responsive proteins such as heat shock proteins, pathogen-related proteins, protein kinases, ascorbate peroxidase, osmotin, ornithine decarboxylase, and some transcription factors, have been detected in some major crops which are thought to give them the ability of withstanding against salt stress. Proteomic analysis of medicinal plants also revealed that alkaloid biosynthesis related proteins such as tryptophan synthase, codeinone reductase, strictosidine synthase, and 12-oxophytodienoate reductase might have major role in production of secondary metabolites. In this review we are comparing some different or similar proteomic responses of several crops and medicinal plants to salt stress and discuss about the future prospects.

Keywords: crops, medicinal plants, proteomics, salt-responsive proteins, secondary metabolites

INTRODUCTION

Salt stress limits agricultural production throughout the world and is becoming an increasingly global problem which affects about 20% of global irrigated land (Flowers and Yeo, 1995). Many major crops such as pepper, eggplant, potato, lettuce, and cabbage are salt-sensitive (Shannon and Grieve, 1999). In addition, important cereals such as rice and maize are also sensitive to hyperosmotic stresses and their production seriously decreases in saline soils (Ngara et al., 2012). Therefore increasing of soil salinization as well as the growing world population shows the increasing need to develop crops which are able to adapt to salt stress.

Tolerance against salt stress needs profound changes in gene expression which is accompanied with changes in composition of plant transcriptome, metabolome, and proteome. Changes in gene expression at transcript level cannot exactly show the changes at protein level. This reflects the high importance of plant proteome since proteins are directly involved in plant stress response. In addition to enzymes, proteins include components of transcription and translation machinery therefore they can regulate plant stress response at transcript and protein levels (Kosova et al., 2011). Thus, investigation of plant response to stress conditions at protein level can provide a powerful tool to reveal the physiological mechanisms underlying plant stress tolerance.

One of the most popular methods to study of the plant responses to environmental stresses is proteome analysis since the extraction of proteins is easy and the obtained two-dimensional electrophoresis gels have great reproducibility and also the mass spectrometry (MS) for sequencing of proteins are very sensitive (Komatsu et al., 2003). Proteomics has been used to study of the expression of salt stress related proteins in several crops such as rice (Parker et al., 2006), potato (Aghaei et al., 2008a), soybean (Aghaei et al., 2008b), and *foxtail millet* (Veeranagamallaiah et al., 2008), which can provide a better indication of cellular activities under salt stress. According to previous studies stress proteins such as osmotin (Qureshi et al., 2007), reactive oxygen species (ROS) scavenging enzymes (Abbasi and Komatsu, 2004), and pathogen-related proteins (PRPs; Dani et al., 2005) might be used as important molecular markers for the improvement of salt tolerance. Zhang et al. (2012) have summarized 2171 salt-responsive proteins from proteomic analysis of 34 different plant species (19 crops and other plants) and functionally categorized these proteins as follows; photosynthesis, carbohydrate and energy metabolism, metabolism, stress and defense, transcription, protein synthesis, protein folding and transport, protein degradation, signaling, membrane and transport, cell structure, cell division/differentiation and fate, miscellaneous, and unknown function. We have discussed the proteomic responses of 13 economically important crop plants to salt stress and also in compare to five medicinal plants.

Medicinal plants are among the major and important group of crops (Rehm and Espig, 1991) which have been used for traditional prevention and treatment of diseases and using herbal medicines have a long history (Williamson, 2003). Based on the World Health Organization (WHO), about 80% of the world populations still

rely on medicinal herbs. Herbal medicinal products are used by nearly 19% of the adult populations in the United States (Kennedy, 2005; Patwardhan et al., 2005); however, the quality and the quantity of secondary metabolites of medicinal plants strongly depend on environmental conditions.

Although the effects of salt stress on crops have been investigated widely and well studied, however, in the case of medicinal plants there is lack of information. The molecular mechanisms of salt tolerance and secondary metabolism in these commercially important crops have not been investigated as other crops. Thus comparing the responses of medicinal plants to salt stress with some other important crops has a great value.

Salt stress significantly affects the production of essential oils and the constituents of medicinal plants, thus the investigation on the mechanisms of salt tolerance in medicinal plants has a great importance. Creation of salt-tolerant medicinal plant leads to increased production of raw materials for drugs, flavors, fragrances, and spices all over the world. In order to increase the production of a special compound in a medicinal plant it is necessary to know which protein or proteins are involved in the biosynthetic pathway and as a consequence, the proteomic approach is a powerful tool to determine the responsible proteins in secondary metabolism in medicinal plants.

Proteomics is also widely used to analyze biochemical pathways and the complex responses of plants to abiotic stresses. Using comparative proteomic investigations of plants before and after specific stresses we are able to reveal the defensive mechanisms which are applied by plants (Timperio et al., 2008). Because of the importance of salt stress effects on crops and medicinal plants and the ability of proteomics as a widely used and powerful tool to determine the proteins which are likely responsible in salt stress responses of these plants in this review we outline the proteome analysis of crops and medicinal plants under salt stress.

EFFECTS OF SALT STRESS ON CROPS

Salt stress results in impairments in growth of all plants as well as crops, and its effects can be categorized in two short and long terms. Osmotic stress, lowering of external water potential and reduction of water uptake by plants, similar to the situation under drought, all fall in the category of short term effects (Wang et al., 2003); however, ion toxicity occurring when crops are not able to compartmentalize ions properly, falls in the long term effects. In salt-tolerant crops leaf cells can remove Na^+ as well as Cl^- from the cytoplasm and then sequester them in the vacuole. Na^+ and Cl^- are easily taken up when salt concentration in the soil increases which consequently leads to displacement of mineral nutrients such as K^+, Ca^{++}, and also nitrate, which can negatively affect the survival of crops. Introduction of ion excess into the cells causes formation of ROS such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, which can disrupt cellular homeostasis and trigger the expression of genes involved in defense mechanisms (Timperio et al., 2008; Du et al., 2010). In addition, gene expression is also influenced by salt stress. For example genes involved in the metabolic pathways of nitrogen reduction and fixation and methionine biosynthesis are significantly affected by salt stress (Ouyang et al., 2007). At higher levels of salt stress, Na^+ and Cl^- have direct toxic effects on the structure of membrane and enzyme leading to the uncontrolled entrance or efflux of minerals and nutrients (Qureshi et al., 2007).

To reveal the facts concerning the proper function of genes and the biochemical kinetics of plants against salt stress, genome sequence information alone is insufficient and consequently it is not possible to determine the exact responsive mechanisms. To solve these problems, more comprehensive approaches including, quantitative and qualitative analysis of gene expression products are necessary at the transcriptome, metabolome, and proteome levels. Many investigations have shown that, some environmental stresses which cause cellular dehydration, like freezing, salt and water stress, often lead to similar changes in plant gene expression and metabolism (Rabbani et al., 2003) and a strong cross-talk can be seen in their signaling pathways. By detection of proteins which are involved in salt stress responses the designated defense mechanism can be inferred (Timperio et al., 2008).

HOW CROPS RESPOND TO SALT STRESS

Responses of crops to salt stress closely depend on their ability of stress tolerance or sensitivity. Classification of crops, based on salt tolerance showed that; sugar beet and durum wheat are salt-tolerant, broad bean, maize, potato, sunflower, and tomato are regarded as moderately salt-sensitive (Katerji et al., 2000). The classification of soybean in two different classes as moderately salt-sensitive and moderately salt-tolerant can be ascribed to difference in variety (Katerji et al., 2000).

Increasing of the concentrations of NaCl in soil results in osmotic stress which leads to create a misbalance in intracellular ion homeostasis (Chinnusamy et al., 2005). As a consequence of osmotic stress the osmotic adjustment of cell cytoplasm will be induced which leads to accumulation of several low-molecular osmolytes such as; raffinose, glycine betaine, and proline as well as high molecular hydrophilic proteins from late embryogenesis abundant proteins superfamily (Kosova et al., 2011). Glycerol, sucrose, polyamines, putrescine, and other molecules which are not highly charged but polar and highly soluble, have a larger hydration shell to protect biological macromolecules against the damaging effects of salt stress (Sairam and Tyagi, 2004). Accumulation of compatible solutes in the plant tissue is a strategy to combat salinity in sugar beet (Wakeel et al., 2011) and some other crops (Zhu, 2002). Free proline plays a major role in the adjustment of the osmotic potential in a number of crops such as soybean (Aghaei et al., 2008b), potato (Aghaei et al., 2008a), and tomato (Amini et al., 2007) under hyperosmotic conditions. Proline acts both as a protective agent and a free-radical scavenger therefore, crops may overproduce proline in an attempt to regulate the pH of their cytosol (Razavizadeh et al., 2009).

Expression of biosynthetic enzymes is an important way to counter salt stress by increasing compatible solute production (Sakamoto and Murata, 2000) and the enhancement of Na^+ export from cells and thereby establishment of homeostasis is the other approach against salinity. Zhu (2001) also showed that overexpression of a plasma membrane Na^+/H^+ antiporter confers salt tolerance in *Arabidopsis*. Maintaining low cytosolic salt concentrations in crops can be controlled by the ability to selective ion uptake, ion exclusion, and compartmentalization of Na^+ in

vacuoles (Liu et al., 2012). Excess toxic ions, for example, Na^+, in the cytosol and a deficiency of essential ions such as K^+ which is a consequence of ionic stress, disrupts ion homeostasis in plant cells (Zhu, 2002) therefore various ion transporters, pumps, and channels play crucial roles in these processes (Zhu, 2003; Reddy and Reddy, 2004).

Antioxidant enzymes and soluble proteins in the cytoplasm of potato (Aghaei et al., 2009) and cotton (Gossett et al., 1994) are able to protect cells from salt-induced oxidative stress. In addition to above mentioned mechanisms which crops use as a strategy against salt stress it has been shown that in tomato, plant growth regulators such as abscisic acid (ABA) and ethylene, also play a pivotal role in the complicated story of abiotic stress (Ouyang et al., 2007).

WHICH PROTEINS ARE INVOLVED IN CROPS IN RESPONSE TO SALT STRESS?

Plant cells may alter their gene expression in order to tolerate salt stress which results in an increase, decrease, induction or total suppression of some stress responsive proteins such as malate dehydrogenase (NADP+) and pyruvate phosphate dikinase (Ngara et al., 2012). Different families of proteins which have been newly synthesized, accumulated, or decreased are known to be associated with crops response to salt stress (**Table 1**). These proteins may be involved in signaling, translation, host-defense mechanisms, carbohydrate metabolism, and amino acid metabolism. Other proteins such as antioxidant enzymes and chaperonins may encounter salt stressors directly and other groups of proteins like key enzyme in osmolyte synthesis encounter salt stress indirectly (Wang et al., 2004). For these reasons elucidating the various mechanisms of plant response to salt stress and their roles in acquired stress tolerance in crops is of great importance.

Over-expression of *ornithine decarboxylase* gene in carrot cells indicated that these cells were significantly more tolerant to salt stress (Bohnert et al., 1995). It was also demonstrated that salt-tolerant varieties of rice showed higher levels of ABA-responsive proteins in their roots (Moons et al., 1995). Because of the important roles of proteins in salt stress tolerance in crops analysis of proteins under salt stress has a great value. Therefore proteome analysis which is an important approach has been recently used to investigate the responses of crops to salt stress as well as many other biotic and abiotic stresses.

In a comparative study of salt-resistant and salt-sensitive wheat genotypes, Wang et al. (2007) identified 23 variety-specific salt-responsive proteins. A major group of proteins which increased under salinity in rice were ROS scavenging enzymes such as: ascorbate peroxidase, dehydroascorbate reductase, peroxiredoxin, superoxide dismutase. Increasing of ROS scavenging enzymes suggests that salt stress results in oxidative stress in rice (Salekdeh et al., 2002; Abbasi and Komatsu, 2004). It has been shown that in pea cultivars exposed to NaCl, leaf mitochondrial Mn-SOD, and chloroplast Cu/Zn-SOD activities increased under salt stress (Hernandez et al., 1995).

Table 1 | Identified major proteins increased in crop plants under salt stress using proteomics.

No.	Crop plant	Identified protein	Role of protein in salt tolerance	Reference
1	Carrot	Ornithine decarboxylase	Proline biosynthesis	Bohnert et al. (1995)
2	Rice	ABA-responsive proteins	ABA biosynthesis	Moons et al. (1995)
3	Rice	APX, DHAR, SOD	ROS scavenging	Abbasi and Komatsu (2004)
4	Rice	Sti1(HSPs)	Defense mechanisms	Kurek et al. (2002)
5	Pea	Mn-SOD	ROS scavenging	Qureshi et al. (2007)
6	Pea	PRPs	Defense mechanisms	Qureshi et al. (2007)
7	Potato	Osmotin-like protein	Osmotic stress tolerance	Aghaei et al. (2008a)
8	Wheat	Glutamine synthase	Protein biosynthesis	Caruso et al. (2008)
9	Wheat	Glycine dehydrogenase	Protein biosynthesis	Caruso et al. (2008)
10	Tobacco	PRPs	Defense mechanisms	Dani et al. (2005)
11	Tobacco	Osmotin	Osmotic stress tolerance	Qureshi et al. (2007)
12	Soybean	LEA proteins	Seed development	Aghaei et al. (2008b)
13	Tomato	NHX1	Ion transport	Ward et al. (2003)
14	Maize	NHX1	Ion transport	Neubert et al. (2005)
15	Sorghum	Malate dehydrogenase, APX	ROS scavenging	Ngara et al. (2012)
16	Sugar beet	Osmotin-like protein	Osmotic stress tolerance	Hajheidari et al. (2005)
17	Sugar beet	Glycine decarboxylase	Metabolism	Wakeel et al. (2011)
18	Sugar beet	Ferredoxin-NADP-reductase	Photosynthesis and metabolism	Wakeel et al. (2011)
19	Sugar beet	Aminomethyltransferase	Metabolism	Wakeel et al. (2011)

DHAR, dehydroascorbate reductase; HSPs, heat shock proteins; PRPs, pathogen-related proteins; LEA, late embryogenesis-abundant; NHX1, Na^+/H^+ antiporter; APX, ascorbate peroxidase; SOD, super oxide dismutase; ABA, abscisic acid.

Nucleoside diphosphate kinase and guanine nucleotide-binding protein which are involved in nucleotide metabolism and enoyl-ACP reductase involving in fatty acid metabolism in rice have been increased under salt stress (Dooki et al., 2006). GTP-binding protein, lectin-like protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activase, ferritin, fructose bisphosphate aldolase, photosystem II oxygen-evolving complex protein, oxygen-evolving enhancer (OEE) protein 2, and SOD were increased in rice (Abbasi and Komatsu, 2004). PRPs, phytochelatins, chaperonins, and metallothioneins were also involved in responses to salt stress in pea (Qureshi et al., 2007). In wheat (*Triticum durum*) it has been shown that, glutamine synthase which is a key enzyme for proline biosynthesis and glycine dehydrogenase which is crucial for glycine betaine biosynthesis were significantly increased under salinity stress (Caruso et al., 2008).

Using proteomic approaches, it has been indicated that chitinases, which are PRPs, were accumulated in leaf apoplast of tobacco plants under saline conditions (Dani et al., 2005). It has been confirmed that in transgenic variety of rice, protein sti1 which is one of the heat shock proteins, appeared to be increased in response to salt stress (Kurek et al., 2002; Qureshi et al., 2007). These proteins are among well known stress responsive proteins which are expressed in response to abiotic stresses such as heat, cold, drought, salinity, and oxidative stress (Wang et al., 2004). Aggregation of stress-denatured proteins is prevented by heat shock proteins, and they facilitate the refolding of proteins in order to restore their native biological functions (Wang et al., 2004).

Oxidative stress tolerance related proteins, glycine betaine synthesis, photosynthesis, adenosine triphosphate (ATP) production, protein degradation, cyanide detoxification, and chaperone activities were increased in *Suaeda aegyptiaca* leaves (Askari et al., 2006). According to Aghaei et al. (2008b) beta-conglycinin and late embryogenesis-abundant protein were increased in soybean root and hypocotyl when exposed to salt stress.

It seems that in transgenic *Arabidopsis* and tomato plants vacuolar Na^+/H^+ antiporter could confer salt tolerance (Ward et al., 2003). The over-expression of NHX1 contributed to improve salt-resistance in maize (Neubert et al., 2005). A novel ethylene responsive factor called TERF1 was identified by Huang et al. (2004) in tomato, which might have function as a link between the ethylene and osmotic stress pathways (Huang et al., 2004). Enoyl-CoA hydratase and phosphoglycerate mutase-like protein were increased in tomato under salt stress (Amini et al., 2007). In sorghum leaf, it has been shown that the majority of the identified salt stress responsive proteins (28 spots) were energy related proteins (50.9%). RuBisCO subunits, sedoheptulose bisphosphatase, phosphoribulokinase, ribulose 5-phosphate isomerase, OEE1, malate dehydrogenase, and ascorbate peroxidase showed an increased abundance following salt stress (Ngara et al., 2012). Osmotin is one of the most studied proteins that are accumulated in salt adaptation (Qureshi et al., 2007). The osmotin gene is responsible for osmoprotectant synthesis, the most important of which are proline and glycine betaine (Holmstrom et al., 2000). Using a proteomic approach, Hajheidari et al. (2005) in sugar beet and Aghaei et al. (2008a) in potato found an osmotin-like protein increased in drought and salt conditions. It seems that osmotin like proteins as well as osmotin can confer salt tolerance to these crops in response to osmotic stress resulted from salt stress. Using proteomic approach it has been proposed that, NADP reductase, aminomethyltransferase, and decarboxylase subunit T in sugar beet have been increased in response to salt stress (Wakeel et al., 2011) which suggests that, these proteins can be considered as salt-responsive proteins in this crop. In addition to proteins which have been increased in salt stressed crops, RuBisCO small subunits, phosphor glycerate kinase, which catalyses a reduction step in Calvin cycle, and phosphoribulokinase, which catalyses regeneration of primary CO_2 acceptor RuBP, were all decreased in salt-treated crops such as soybean (Sobhanian et al., 2010) and *Triticum durum* (Caruso et al., 2008).

Regarding above mentioned literature about different proteins which have been induced under salt stress in different crops it can be suggested that salt-responsive proteins are involved in a variety of metabolic processes, such as scavenging of ROS, signal transduction, transcription and translation, transporting, chaperones, cell wall biosynthesis, photosynthesis, processing and degradation, metabolism of energy, amino acids, and hormones.

EFFECTS OF SALT STRESS ON MEDICINAL PLANTS

Medicinal and aromatic plants are cultivated because of their active constituents which are used for different purposes, especially for production of anticancer drugs such as vincristine and paclitaxel (Verpoorte et al., 2002). In order to create high yielding genotypes of these plants under different environmental conditions, response of medicinal and aromatic plants to salinity stress has been reported in a substantial number of literatures (Said-Al Ahl and Omer, 2011).

Salt stress affects medicinal plants on different physiological stages. One of the most salt-sensitive growth stages which is severely inhibited with increasing salinity is seed germination stage (Sosa et al., 2005). Seed germination of *Ocimum basilicum* (Miceli et al., 2003), *Petroselinum hortense* (Ramin, 2005), sweet marjoram (Ali et al., 2007), and *Thymus maroccanus* (Belaqziz et al., 2009) showed significant decrease under salt stress. Another stage which is negatively influenced by salinity is seedling growth. It has been previously reported that, seedling growth of *Thymus maroccanus* (Belaqziz et al., 2009), basil (Ramin, 2005), chamomile and marjoram (Ali et al., 2007) were severely decreased under salt stress. Slow or less mobilization of reserve foods, suspending the cell division, enlarging and injuring hypocotyls which are induced by salt stress have been proposed as main reasons for these effects (Said-Al Ahl and Omer, 2011).

Morphological characteristics such as number of leaves, leaf area, and leaf biomass have been reduced under salt stress in a number of medicinal plants. Similarly, negative effects of salt stress have been observed in *Majorana hortensis* (Shalan et al., 2006), peppermint (Aziz et al., 2008), geranium (Leithy et al., 2009), *Thymus vulgaris* (Najafian et al., 2009), sage (Ben Taarit et al., 2009), and *Mentha pulegium* (Queslati et al., 2010). Increasing salt stress pronouncedly increased total soluble carbohydrate contents of *Salvia officinalis* (Hendawy and Khalid, 2005) and *Satureja hortensis* (Najafi et al., 2010).

There are some common responses between medicinal plants and crops in response to salt stress and the mechanisms of salt

tolerance in medicinal plants include many of the mechanisms which have been explained for crops. Synthesis of compatible solutes such as proline (Ghoulam et al., 2002), nitrogen-containing compounds such as amino acids, amides, proteins, polyamines (Mansour, 2000), and soluble sugars such as glucose, fructose, sucrose, and fructans, have been reported in medicinal plants under salt stress (Omami et al., 2006). It is supposed that these compounds function in protection of cellular macromolecules, scavenging of free radicals, osmotic adjustment, storage of nitrogen, and maintenance of cellular pH (Said-Al Ahl and Omer, 2011). However, because of the presence of high concentrations of different aromatic compounds in these plants such as isoprenoids, phenols, or alkaloids, at least parts of their response to salt stress is mediated by antioxidant properties of these aromatic compounds. Phenylpropanoid derived phenols such as flavonoids, tannins, and hydroxycinnamate esters, which are produced in the course of various stress situations, represent important radical scavengers (Selmar, 2008).

SALT STRESS AND SECONDARY METABOLISM IN MEDICINAL PLANTS

Secondary metabolites are supposed to have several functions, e.g., protective role against herbivores or pathogens, or an attracting role for pollinators and seed spreading animals in medicinal plants (Selmar, 2008). The close relationship between plant secondary metabolism and defense response is widely recognized (Vasconsuelo and Boland, 2007). Alkaloids, anthocyanins, flavonoids, quinones, lignans, steroids, and terpenoids are among plant secondary metabolites which have found commercial applications as drug, dye, flavor, fragrance, insecticide, and antioxidants (Jacobs et al., 2000; Verpoorte et al., 2002). Therefore secondary metabolites of medicinal plants have a great value; however, molecular mechanisms underlying their production have not been investigated widely.

Production of secondary metabolites by plants is not always satisfactory and there are several factors which can restrict their production. Type of medicinal plant species or genus, particular growth or developmental stage, specific seasonal conditions, nutrient availability, or stress conditions are among these factors (Verpoorte et al., 2002). It has been shown that reactions to salt and drought stress might be responsible for the increase or decrease in the content of relevant natural products; however, scientific background in this field is still rare (Selmar, 2008). Medicinal plants under salt stress similar to drought conditions accumulate higher concentrations of secondary compounds than control plants which are cultivated under standard conditions. Selmar (2008) monitored a strong increase in the concentration of tropane alkaloids in *Datura inoxia* plants under salt stress.

Essential oils are among important secondary metabolites in medicinal plants. Contradictory reports have been found concerning the response of medicinal plants in terms of essential oil production to salt stress. Essential oil yield decreased under salt stress in *Trachyspermum ammi* (Ashraf and Orooj, 2006). Similar results have been reported in several medicinal plants, e.g., *Mentha piperita* (Tabatabaie and Nazari, 2007), *Thymus maroccanus* (Belaqziz et al., 2009), basil (Said-Al Ahl and Omer, 2011), and apple mint (Aziz et al., 2008). Although the main essential oil constituents in *Matricaria recutita* increased under salt stress (Baghalian et al., 2008); however, in *Origanum vulgare* the content of carvacrol (the main essential oil constituent) and p-cymene and γ-terpinene decreased under salt stress (Said-Al Ahl and Omer, 2011). Phenolic acid concentration increased in spearmint (Al-Amier and Craker, 2007), *Achillea fragrantissima* (Abd EL-Azim and Ahmed, 2009), and *Mentha pulegium* (Queslati et al., 2010) as a result of salt stress. These contradictory results represents the different responses of medicinal plants to salt stress regarding to their essential oil production; however, the fact that, some medicinal plants may increase the production of essential oil concentration or its main constituents in response to salt stress encourage us to determine the molecular mechanisms of salt stress on the production of secondary metabolites in medicinal plants.

PROTEOMIC ANALYSIS OF MEDICINAL PLANTS TO INVESTIGATE THE PRODUCTION OF SECONDARY METABOLITES

Technical developments in genomics, proteomics, and metabolomics represent a challenging complexity for scientific analysis and will open new perspectives for ethno botanical and phytomedical research purposes. Proteomics provides a promising approach for studying secondary metabolism in plants and plant cells. Unfortunately the natural yield of secondary metabolites in medicinal plants is generally low and the biochemistry of the biosynthesis of these compounds is too complicated which is poorly understood. Cell suspension cultures and metabolic engineering are among strategies to increase the yield of these commercially important chemicals. Over-expression of rate limiting enzymes which are involved in biosynthesis of these compounds has been used for this purpose (Verpoorte et al., 1999). Therefore it is necessary to identify proteins involved in the secondary metabolites biosynthesis. Enzyme isolation and characterization by current approaches is time consuming and troublesome, thus, the proteomic approach is a faster and more complete and using this technology we are allowed to identify regulatory and transport proteins as well as enzymes.

Table 2 summarizes recent proteomic investigations on some medicinal plants and represents some of proteins which are supposed to involve in the production of secondary metabolites. The medicinal plant *Catharanthus roseus* has been used as a best studied model system for secondary metabolite production (Verpoorte et al., 1997). This plant produces some effective anti-tumor drugs, vinblastine and vincristine which are alkaloid compounds. However, alkaloid yields in suspension cell cultures are generally too low to allow commercialization. Jacobs et al. (2000) has used two-dimensional gel electrophoresis for proteomic investigations of alkaloid production in *C. roseus*. Influence of zeatin and 2,4-dichlorophenoxyacetic acid (2,4-D) on protein patterns and alkaloid accumulation of *C. roseus* in a proteomic approach showed that, proteins, which were decreased by 2,4-D and increased by zeatin, may have a direct function as an enzyme or an indirect role as a regulatory or transport protein in alkaloid biosynthesis. A 28 kDa polypeptide which increased by zeatin showed a close correlation with alkaloid production (Jacobs et al., 2000). Another proteome analysis of *C. roseus* resulted in the differential expressions of 88 protein spots which were selected for

Table 2 | Identified proteins and their role in secondary metabolism in medicinal plants using proteomic approach.

No.	Medicinal plant	Identified protein	Role of protein in secondary metabolism	Reference
1	*Catharanthus roseus*	Strictosidine synthase	Biosynthesis of strictosidine in alkaloid biosynthesis	Jacobs et al. (2005)
2	*Catharanthus roseus*	Tryptophan synthase	Biosynthesis of tryptamine as alkaloid precursor	Jacobs et al. (2005)
3	*Catharanthus roseus*	12-oxophytodienoate reductase	Biosynthesis of the regulator jasmonic acid	Jacobs et al. (2005)
4	*Panax ginseng*	Enolase, glyceraldehyde 3-phosphate dehydrogenase, aldolase	Ginsenoside biosynthesis	Nam et al. (2005)
5	*Chelidonium majus*	Disease/defense-related proteins Nucleic acid binding proteins	Secondary metabolite metabolism	Nawrot et al. (2007)
6	*Papaver somniferum*	Codeinone reductase	Morphine biosynthesis	Decker et al. (2000)

identification by MS. Full protein spots were identified including two isoforms of strictosidine synthase, which catalyzes the formation of strictosidine in the alkaloid biosynthesis; tryptophan synthase, which is needed for the supply of the alkaloid precursor tryptamine, 12-oxophytodienoate reductase, which is indirectly involved in the alkaloid biosynthesis as it catalyzes the last step in the biosynthesis of the regulator jasmonic acid (Jacobs et al., 2005). Jasmonic acid is essential for signal transduction in the production process of alkaloids. Its involvement in stress reactions was demonstrated by induction of defense proteins including proteins involved in the biosynthesis of secondary metabolites. Jasmonic acid induces alkaloid biosynthesis in *C. roseus* (Jacobs et al., 2005).

Effects of the secondary metabolites of *Salvia miltiorrhiza* on atherosclerotic lesions (Hung et al., 2010) and cancer have been investigated at the proteomic level (Buriani et al., 2012). Another medicinal plant which has been analyzed by proteomic approach is *Panax ginseng*. The important secondary metabolites of ginseng especially ginsenoids are mainly produced in its roots. In a proteomics experiment in order to find the proteins which are involved in production of its important secondary metabolites, the hairy roots of *Panax ginseng* were been cultured and the root proteome was analyzed. From the 159 cultured hairy root proteins in ginseng, the putative functions of 91 proteins were ascertained. More than 20% of the identified proteins were related to energy metabolism and stress response. Although some proteins such as enolase, glyceraldehyde 3-phosphate dehydrogenase, and aldolase were identified as isotypes; however, none of them was directly related to the metabolism of secondary products (Nam et al., 2005).

In order to determine the major protein content of medicinal plant *Chelidonium majus*, two-dimensional gel electrophoresis of milky sap was investigated. Among 21 protein spots, defense related proteins, nucleic acid binding proteins as well as signaling proteins comprised the main protein components of milky sap in this plant (Nawrot et al., 2007). However, none of identified proteins in this plant could be directly related to its secondary metabolites production. In another attempt, to determine which proteins are involved in production of morphine or other secondary metabolites in opium poppy Decker et al. (2000) performed a proteomic analysis using two-dimensional gel electrophoresis of poppy latex. The main detected protein was codeinone reductase which suggests that codeinone reductase might be a specific enzyme in morphine biosynthesis in this important medicinal plant.

The main limitation for the identification of proteins from medicinal plants is the lack of sequence data of both genes and proteins. For example, the SWISS-PROT database still contains only a few protein entries from *C. roseus* (Jacobs et al., 2005), *Panax ginseng* (Nam et al., 2005), *Papaver somniferum* (Decker et al., 2000), and many other medicinal plants. Consequently the lack of genomic DNA sequence and protein information constitutes a major bottleneck with regard to the identification of proteins in proteomic studies of medicinal plants.

FUTURE PROSPECTIVE

Among abiotic stresses, salt stress is a well studied stress in which proteomic based techniques have provided a powerful tool to reveal the molecular mechanisms of salt stress responses. Several salt-responsive proteins have been proposed for crops using these techniques and using these proteins and their corresponding genes it will be possible to change salt-sensitive crops to salt-tolerant crops in near future. Despite these advances in crop stress tolerance researches using proteomics, there is still a few and limited investigations in the field of proteome analysis of medicinal plants under salt stress or other situations. Although medicinal plants are very important regarding to their secondary metabolites which are used as herbal drugs, flavors, fragrances, spices, and other purposes, the molecular mechanisms underlying the production of these compounds still remained unclear. There are several pharmaceuticals on the market that are highly expensive due to the fact that these compounds are only found in rare plants and often in extreme low concentrations. Podophyllotoxin (Jusling et al., 2006) and paclitaxel (Verpoorte et al., 2002) are clear examples of pharmaceuticals that can only be produced through the isolation from plants. To achieve a sustainable source of such compounds scientists all over the world have been experimenting with biotechnological approaches aiming at the development of an alternative production system. However, metabolite engineering strategies in this field are time consuming and troublesome.

Proteomics technology has proven its success in many fields of plant sciences, thus in the field of phytochemistry, proteomic approaches have been followed and changes in protein expression could be correlated with the accumulation of secondary metabolites. In spite of a few successes in proteomic analysis of some

medicinal plants, the results obtained so far make it clear that proteomics still has to fulfill much of its presumed potential in this area. Although proteomic techniques are rapidly developing; however, some practical constraints still remain which are mainly associated with sample preparation and protein identification. In addition to these problems, one of the major limitations in this field is the lack of genome information about medicinal plants. Many of detected proteins in proteomic analysis of medicinal plants have been remained unidentified. Therefore advances in genome-based information about medicinal plants will lighten new horizons in the field of medicinal plants proteomics. We hope that in near future identification of key proteins involved in the production of secondary metabolites in medicinal plants will leads to increased production of highly appreciated anticancer compounds like vincristine, paclitaxel (Verpoorte et al., 2002), vinblastine (Verpoorte et al., 1997), podophyllotoxin (Jusling et al., 2006), and other valuable herbal drugs which are critical for human beings health all over the world.

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Contribution of proteomic studies towards understanding plant heavy metal stress response

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Modulation of plant proteome composition is an inevitable process to cope with the environmental challenges including heavy metal (HM) stress. Soil and water contaminated with hazardous metals not only cause permanent and irreversible health problems, but also result substantial reduction in crop yields. In course of time, plants have evolved complex mechanisms to regulate the uptake, mobilization, and intracellular concentration of metal ions to alleviate the stress damages. Since, the functional translated portion of the genome plays an essential role in plant stress response, proteomic studies provide us a finer picture of protein networks and metabolic pathways primarily involved in cellular detoxification and tolerance mechanism. In the present review, an attempt is made to present the state of the art of recent development in proteomic techniques and significant contributions made so far for better understanding the complex mechanism of plant metal stress acclimation. Role of metal stress-related proteins involved in antioxidant defense system and primary metabolism is critically reviewed to get a bird's-eye view on the different strategies of plants to detoxify HMs. In addition to the advantages and disadvantages of different proteomic methodologies, future applications of proteome study of subcellular organelles are also discussed to get the new insights into the plant cell response to HMs.

Keywords: antioxidant, heavy metal, HSPs, phytochelatins, proteomics, PR protein

INTRODUCTION

High-throughput OMICS techniques are extensively being exploited in recent times to dissect plants molecular strategies of heavy metals (HMs) stress tolerance. Plants growing in HMs contaminated environment have developed coordinated homeostatic mechanisms to regulate the uptake, mobilization, and intracellular concentration of toxic metal ions to alleviate stress damages. As the functional translated portion of the genome play a key role in plant stress response, proteomic studies provide us a finer picture of protein networks and metabolic pathways primarily involved in cellular detoxification and tolerance mechanism against HM toxicity.

By definition, elements having specific gravity above five are considered as HMs. Nevertheless, the term HM commonly refers to toxic metals, e.g., cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), zinc (Zn) as well as hazardous metalloids viz., arsenic (As), boron (B), which exert negative effects on plant growth and development (Hossain et al., 2012a).

Most of the HMs get their entry into plant root system via specific/generic ion carriers or channels (Bubb and Lester, 1991). The lack of specificity of transporters that are primarily involved in uptake of essential elements such as Zn^{2+}, Fe^{2+}, and Ca^{2+} allow the entry of Cd^{2+}, Pb^{2+} (Welch and Norvell, 1999; Perfus-Barbeoch et al., 2002). Once HM ions enter the cell, cellular functions are affected by a wide range of actions. The negative impact of HM includes binding of HM ions to sulfhydryl groups of proteins, replacement of essential cations from specific binding sites, leading to enzyme inactivation and production of reactive oxygen species (ROS), resulting in oxidative damages to lipids, proteins and nucleic acids (Sharma and Dietz, 2009).

Over the last decade, extensive research on plants response to HM stress has been conducted to unravel the tolerance mechanism. Genomics technologies have been useful in addressing plant abiotic stress responses including HM toxicity (Bohnert et al., 2006). However, changes in gene expression at transcript level have not always been reflected at protein level (Gygi et al., 1999). An in-depth proteomic analysis is thus of great importance to identify target proteins that actively take part in HM detoxification mechanism.

Plant response to HM stress has been reviewed extensively over the past decade (Sanita Di Toppi and Gabbrielli, 1999; Cobbett, 2000; Ma et al., 2001; Cobbett and Goldsbrough, 2002; Hall, 2002; Maksymiec, 2007; Sharma and Dietz, 2009; Verbruggen et al., 2009; Yang and Chu, 2011; Hossain et al., 2012a). However, review articles on application of proteomics in analyzing cellular mechanism for HM tolerance are limited (Ahsan et al., 2009; Luque-Garcia et al., 2011; Villiers et al., 2011).

Current review represents the state of art of recent developments in proteomic techniques and significant contributions made so far to strengthen our knowledge about plants HM-stress

Abbreviations: CBB, coomassie brilliant blue; 2-DE, two-dimensional polyacrylamide gel electrophoresis; GS, glutamine synthetase; GSH, glutathione; GST, glutathione *S*-transferase; IEF, isoelectric focusing; LC, liquid chromatography; MS, mass spectrometry; MTs, metallothioneins; PCs, phytochelatins; pI, isoelectric point; PR, pathogenesis related; ROS, reactive oxygen species; SOD, superoxide dismutase.

response cascade at protein level. Special emphasis is given to highlight the role of metal stress-related proteins engage in HM ions sequestration, antioxidant defense system, and primary metabolism for deeper understanding of coordinated pathways involve in detoxification of HM ions within plant cells. Furthermore, future applications of proteome study of subcellular organelles are discussed to get the new insights into the plant cell response to HMs.

QUANTITATIVE PROTEOMIC TECHNIQUES USED FOR ANALYSIS OF HM-RESPONSIVE PROTEINS

Conventional two-dimensional gel electrophoresis (2-DE) approach coupled with protein identification by mass spectrometry (MS) has been the most widely used proteomic technique for investigation of HM-induced alteration of plant proteome composition (**Table 1**). Protein extraction and purification from the HM-stressed tissue is the most crucial step in 2-DE approach, as the amount and quality of the extracted proteins ultimately determine the protein spot number, resolution, and intensity. Phenolic compounds, proteolytic and oxidative enzymes, terpenes, pigments, organic acids, inhibitory ions, and carbohydrates are some common interfering substances present in recalcitrant plant tissues. Inferior 2-D separation results due to proteolytic breakdown, streaking, and charge heterogeneity. Proteomic studies on plant response against HM stress have revealed that trichloroacetic acid/acetone precipitation (Patterson et al., 2007; Zhen et al., 2007; Kieffer et al., 2008; Alves et al., 2011; Hossain et al., 2012b,c) and phenol-based (Bona et al., 2007; Alvarez et al., 2009; Vannini et al., 2009; Lee et al., 2010; Ritter et al., 2010; Rodríguez-Celma et al., 2010; Ahsan et al., 2012; Sharmin et al., 2012) protocols are the effective protein extraction methods for obtaining high quality proteome map. Nevertheless, phenol-based method is the most appropriate in extracting glycoproteins, and produce high-resolution proteome map for recalcitrant plant tissues (Saravanan and Rose, 2004; Komatsu and Ahsan, 2009).

As compared to classical staining procedure of 2-DE gel using CBB or silver staining, advanced fluorescence two-dimensional difference gel electrophoresis (2-D DIGE) proteomic approach is now being used which allows comparison of the differentially expressed proteins of control and HM-stressed tissue on one single gel (Kieffer et al., 2008; Alvarez et al., 2009). DIGE is basically a gel-based method where proteins were labeled with fluorescent dyes (CyDyes – Cy2, Cy3, and Cy5) prior to electrophoresis. With the advancement of technology multiplexed isobaric tagging (iTRAQ) of peptides has allowed comparative, quantitative analysis of multiple samples. This second generation gel free proteomic approach has been well exploited for gaining comprehensive understanding of plants response to Cd and B (Patterson et al., 2007; Alvarez et al., 2009; Schneider et al., 2009).

PLANT STRATEGIES OF HM TOLERANCE

In course of time, higher plants have evolved sophisticated mechanisms to regulate the uptake, mobilization, and intracellular concentration of HM ions (**Figure 1**). Apart from the plasma membrane exclusion method, the most common way to protect the cell from the adverse effects of HMs includes synthesis of membrane transporters and thiol-containing chelating compounds for vacuolar sequestration. Furthermore, increased abundance of defense proteins for effective ROS scavenging and molecular chaperones for re-establishing normal protein conformation help HM-stressed plants to maintain redox homeostasis. Modulations of vital metabolic pathways – photosynthesis and mitochondrial respiration – further help the stressed plant to produce more reducing power to compensate high-energy demand of HM challenged cells.

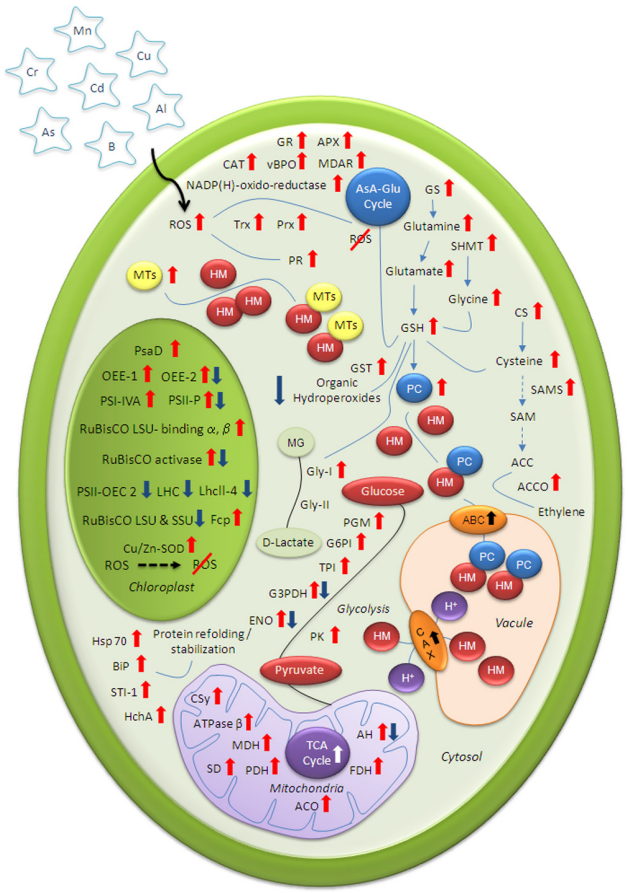

FIGURE 1 | Schematic illustration of various cellular mechanisms for mitigating heavy metal (HM) stress. Information about highlighted proteins gathered from published proteomic articles related to plant HM-toxicity. Up and down arrows indicate HM-induced increase and decrease protein abundance respectively. ATPase β, ATP synthase subunit beta; AH, aconitate hydratase; AsA-Glu, ascorbate glutathione; APX, ascorbate peroxidase; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, aconitase; CAT, catalase; CAX, cation/proton exchanger; CS, cysteine synthase; CSy, citrate synthase; ENO, enolase; FDH, formate dehydrogenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glutathione reductase; Gly-I, glyoxalase I; GS, glutamine synthetase; GSH, reduced glutathione; LHC, light harvesting complex; LhcII-4, light-harvesting chlorophyll-a/b binding protein; LSU, large subunit; MTs, metallothioneins; MG, methylglyoxal; MDAR, monodehydroascorbate reductase; MDH, malate dehydrogenase; OEE, oxygen-evolving enhancer protein; PC, Phytochelatin; Prx, peroxidoxin; PR, pathogenesis-related; PDH, pyruvate dehydrogenase; PSII-OEC 2, photosystem II oxygen-evolving complex protein 2; PS, photosystem; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; SD, succinate dehydrogenase; SAM, S-adenosylmethionine; SSU, small subunit; Trx, thioredoxin; TPI, triosephosphate isomerase; TCA, tricarboxylic acid.

Table 1 | Summary of functional proteomic analyses in response to heavy metal stress (2007–2012).

Metal	Plant (tissue)	Protein extraction buffer + precipitation	Protein solubilization/ lysis buffer	Proteomic methodologies	IP	Major findings	Reference
Cd	*G. max* L. cvs. Harosoy (H), Fukuyutaka (F), CDH-80 (C) (leaf, root)	10% TCA, 0.07% 2-ME in acetone	8 M urea, 2 M thiourea, 5% CHAPS, 2 mM TBP, ampholytes (pH 3–10)	IPG, 2-DE, nanoLC-MS/MS, MALDI-TOF MS	32 (HL), 26 (FL), 44 (CL), 16 (R)	Activation of SOD, APX, and CAT ensures cellular protection from ROS mediated damages under cadmium stress; enhanced expression of molecular chaperones help in stabilizing protein structure and function, thus maintain cellular homeostasis.	Hossain et al. (2012b)
	G. max L. cv. Enrei (leaf)	10% TCA, 0.07% 2-ME in acetone	8 M urea, 2 M thiourea, 5% CHAPS, 2 mM TBP, ampholytes (pH 3–10)	IPG, 2-DE, nanoLC-MS/MS, MALDI-TOF MS	78	High abundance of Hsp70 helps BABA-primed plants to maintain normal protein functions; higher abundance of Prx indicates BABA potentiated antioxidant defense system to combat Cd stress.	Hossain et al. (2012c)
	G. max L. cv. Enrei, Harosoy (root microsome)	0.5 M Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM DTT, 0.25 M sucrose, 1 mM PMSF + Tris–HCl saturated phenol	8.5 M urea, 2.5 M thiourea, 5% CHAPS, 1% DTT, 1% Triton X-100, 0.5% Biolyte (pH 5–8)	IPG, 2-DE, nanoLC-MS/MS	22	Up-regulation of proteins associated with Cd-chelating pathways and increased lignification of xylem vessels lead to low root-shoot translocation of Cd in cv. Enrei.	Ahsan et al. (2012)
	L. esculentum Mill cv. Tres Cantos (root)	phenol-saturated Tris–HCl 0.1 M (pH 8.0), 5 mM ME	8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 2 mM PMSF, 0.2% (v/v) 3–10 ampholytes	IPG, 2-DE, MALDI-TOF-MS, LIFT TOF–TOF	27 (low Cd), 33 (high Cd)	Low Cd treatment (10 μM) activates glycolysis, TCA cycle and respiration; at high Cd (100 μM) major decreases in growth, a shutdown of the carbohydrate metabolism and decreases in respiration takes place.	Rodríguez-Celma et al. (2010)
	O. sativa L. cv.Dongjin (Root, leaf)	0.5 M Tris–HCl (pH 8.0), 50 mM EDTA, 900 mM sucrose, 100 mM KCl, 2% ME, 1 mM PMSF + Tris-buffered phenol (pH 8.0)	7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 50 mM DTT, 0.5% IPG buffer	IPG, 2-DE, MALDI-TOF MS	18 (R) 19 (L)	ROS scavengers (GST, APX, NADH-ubiquinone oxidoreductase) primarily up-regulated in roots under Cd treatment, indicates prompt antioxidative response against oxidative stress damages.	Lee et al. (2010)
	H. vulgare L. var. Baraka (leaf mesophyll tonoplast)	Tonoplast proteins dissolved in iTRAQ dissolution buffer	–	iTRAQ labeling, MALDI-TOF/TOF MS	56	Candidate proteins like CAX1a and MRP-like ABC transporter play significant role in vacular Cd^{2+} transport, hence Cd^{2+} detoxification.	Schneider et al. (2009)

(Continued)

Table 1 | Continued

Metal	Plant (tissue)	Protein extraction buffer + precipitation	Protein solubilization/ lysis buffer	Proteomic methodologies	IP	Major findings	Reference
	B. juncea L. (Acc: PI 173874) (root)	Tris-buffered phenol (pH 8.8) and 600 mL of 0.1 M Tris–HCl with 10 mM EDTA, 0.4% v/v 2-ME, 0.9 M sucrose	DIGE solubilization buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.2% w/v SDS, 10 mM Tris, pH 8.5), and 0.5 M bicine pH 8.4 with 0.09% w/v SDS (for iTRAQ Label)	IPG, 2-D DIGE, iTRAQ, nanoLC-MS/MS	102 (DIGE), 585 (iTRAQ)	*O*-acetylserine sulfhydrylase, glutathione-*S*-transferase and glutathione-conjugate membrane transporter play essential role in the Cd hyperaccumulation and tolerance of *B. juncea*.	Alvarez et al. (2009)
	P. tremula L. (leaf)	20% TCA and 0.1% (w/v) DTT in ice-cold acetone	Labeling buffer	IPG, 2-D DIGE, MALDI-TOF-TOF MS	125	Up-regulation of mitochondrial respiration provides energy and reducing power to cope with metal stress, photosynthesis comparatively less affected.	Kieffer et al. (2008)
Cu	*E. siliculosus* strains Es32, Es524 (algal tissue)	1.5% w/v PVP, 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl (pH 7.5), 250 mM EDTA, protease inhibitor, 2% v/v ME, 0.5% w/v CHAPS + phenol saturated Tris–HCl (pH 7.5)	7 M Urea, 2 M Thiourea, 4% w/v CHAPS, 60 mM DTT, 20 mM Tris–HCl (pH 8.8), Biolytes (pH 3–10)	IPG, 2-DE, MALDI-TOF MS	10 (Es32) 14 (Es524)	Copper stress leads to up-regulation of photosynthesis (PSII Mn-stabilizing protein of OEC33), glycolysis, and pentose phosphate metabolism; higher accumulation of HSP70 and vBPO for proper protein folding and ROS detoxification respectively.	Ritter et al. (2010)
	O. sativa L. Wuyunjing (germinating embryos)	50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM PMSF + ice-cold acetone with 1 mM DTT	8 M urea, 4% CHAPS, 65 mM DTT, 0.2% (w/v) Biolytes (pH 3–10)	IPG, 2-DE, MALDI-TOF MS	16	First proteomic evidence that metallothionein and CYP90D2 (a putative small cytochrome P450) are Cu-responsive proteins in plants.	Zhang et al. (2009)
	C. sativa Var. Felina 34 (root)	0.5 M Tris–HCl (pH 7.5), 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2 mM PMSF/DMSO, 2% v/v ME + phenol saturated Tris–HCl (pH 8.8)	9 M urea, 4% w/v CHAPS, 0.5% Triton X-100, 20 mM DTT, 2% v/v IPG Buffer	IPG, 2-DE, LC-MS/MS	20	Copper induced aldo/keto reductase acts as copper chaperone reduce copper ions to Cu (I), promote PCs-mediated vacuolar transport; Suppression/no change in ROS scavenging enzymes.	Bona et al. (2007)
	O. sativa L. cv. Hwayeong (Germinating seeds)	0.5 M Tris–HCl (pH 8.3), 2% v/v NP-40, 20 mM $MgCl_2$, 2% v/v ME, 1 mM PMSF, 1% w/v PVP + acetone	9.5 M urea, 2% v/v NP-40, and 2.5% v/v pharmalytes (pH 3–10: pH 5–8: pH 4–6.5 = 1:3.5:2.5)	IEF gel (tube gel), 2-DE, MALDI-TOF MS	25	Excess Cu induces oxidative stress thus hampering metabolic processes; up-regulation of antioxidant and stress-related regulatory proteins (glyoxalase I, peroxiredoxin) help to maintain cellular homeostasis.	Ahsan et al. (2007b)

(Continued)

Table 1 | Continued

Metal	Plant (tissue)	Protein extraction buffer + precipitation	Protein solubilization/ lysis buffer	Proteomic methodologies	IP	Major findings	Reference
B	*L. albus* cv. Rio Maior (root)	0.06 M DTT, 10% (w/v) TCA in cold acetone with 0.06 M DTT	2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 0.4% (v/v) TritonX-100, 0.06 MDTT, and 1%(v/v) IPG buffer 3–10 NL	IPG, 2-DE, LC-MS/MS	128	Proteins associated with energy (glycolysis, TCA cycle, oxidation–reduction), cell division, protein metabolic processes suppressed under B deficiency.	Alves et al. (2011)
	H. vulgare cvs. GP, Cp, Sh, Cp x Sh DH (Root, leaf)	50 mM phosphate buffer (pH 7.5), 20 mM KCl, 0.5 M Suc, 10 mM DTT, 0.2 mM PMSF, 10 mM EDTA, 10 mM EGTA + 10% (w/v) TCA in acetone	0.5 M TEAB (pH 8.5) containing 0.1% SDS	iTRAQ peptide tagging, MS/MS	139	Higher abundance of Iron deficiency sensitive2 [IDS2], IDS3, and methylthio-ribose kinase observed in B-tolerant barley is linked to siderophore production	Patterson et al. (2007)
As	*Anabaena* sp. PCC7120 (algal cells)	10 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl + 10% (w/v) TCA in acetone	7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 1.0% IPG buffer (4–7)	IPG, 2-DE, MALDI-TOF, and LC-MS	45	Up-regulations of PGK, FBA II, FBPase, TK, ATP synthase, Prx, Trx, oxidoreductase help to maintain normal glycolysis, PPP, and turnover rate of Calvin cycle, protect cells from oxidative stress, thereby helping As-stress acclimation.	Pandey et al. (2012)
	O. sativa L. cv. Dongjin (leaf)	0.5 M Tris–HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) ME, 1 mM PMSF, 0.7 M sucrose + acetone precipitation	8 M urea, 1% CHAPS, 0.5% (v/v) IPG buffer pH 4–7, 20 mM DTT	IPG, 2-DE, MALDI-TOF MS, ESI-MS/MS	12	Energy and metabolism related proteins over expressed indicating higher energy demand under As stress; down-regulation of RuBisCO and chloroplast 29 kDa ribonucleoproteins lead to decreased photosynthesis.	Ahsan et al. (2010)
As (V and III)	*A. tenuis* (leaf)	Glacial acetone containing 0.07% (v/v) 2-ME, 0.34% (w/v) plant protease inhibitor, and 4% (w/v) PVP	4% (w/v) CHAPS, 7 M urea, 2 M thiourea, 2% (w/v) DTT, 1% (w/v) pharmalytes pH 3–10, 1% (w/v) resolytes pH 6–9.5	IPG, 2-DE, MALDI-TOF MS	31	As treatment resulted in partial disruption of the photosynthetic processes with prominent fragmentation of the RubisCO.	Duquesnoy et al. (2009)
	O. sativa L. cv. Dongjin (root)	0.5 M of Tris–HCl (pH 8.3), 2% v/v NP-40, 20 mM $MgCl_2$, 2% v/v ME, 1 mM PMSF, 0.7 M sucrose + acetone precipitation	8 M urea, 1% CHAPS, 0.5% v/v IPG buffer pH 4–7, 20 mM DTT	IPG, 2-DE, MALDI-TOF MS	23	Energy, primary metabolic pathways suppressed under stress; higher GSH content coupled with enhanced expressions of GR, SAMS, GSTs, CS, GR mitigate As-induced oxidative stress.	Ahsan et al. (2008)
Mn	*V. unguiculata* [L.] Walp. cvs TVu 91, TVu 1987 (leaf)	700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, and 2% v/v ME + water saturated phenol	8 M urea, 2% w/v CHAPS, 0.5% v/v IPG buffer pH 3–11, 50 mM DTT	IPG, 2-DE, Nano-LC-MS/MS, ESI MS/MS	8	Lower abundance of chloroplastic proteins involved in CO_2 fixation and photosynthesis indicate channelizing metabolic energy to combat the Mn-stress; coordinated interplay of apoplastic and symplastic reactions essential for stress response.	Führs et al. (2008)

(Continued)

Table 1 | Continued

Metal	Plant (tissue)	Protein extraction buffer + precipitation	Protein solubilization/ lysis buffer	Proteomic methodologies	IP	Major findings	Reference
Cr	*M. sinensis* cv. Kosung (root)	0.5 M Tris–HCl, pH 8.3, 2% (v/v) NP-40, 20 mM $MgCl_2$, 1 mM PMSF, 2% (v/v) ME, and 1% (w/v) PVP	8 M urea, 1% CHAPS, 0.5% (v/v) IPG buffer pH 4–7, 20 mM DTT	IPG, 2-DE, MALDI-TOF MS, MALDI-TOF/TOF MS	36	Novel accumulation of chromium-responsive proteins (e.g. IMPase, nitrate reductase, adenine phosphoribosyl transferase, formate dehydrogenase, putative dihydrolipoamide dehydrogenase) observed; Cr toxicity is linked to heavy metal tolerance and senescence pathways.	Sharmin et al. (2012)
	P. subcapitata strain Hindák (algal cells)	500 mM Tris–HCl (pH 8), 700 mM sucrose, 10 mM EDTA, 4 mM ascorbate, 0.4% ME, 0.2% Triton X-100 10%, 1 mM PMSF, 1 μM Leupeptin, 0.1 mg/mL Pefabloc + water saturated phenol	7 M urea, 2 M thiourea, 4% CHAPS, 50 mg/mL DTT	IPG, 2-DE, LC-ESI-MS/MS	16	Cr-stress target photosynthetic proteins (RuBisCO, RuBisCO activase, Light Harvesting Chla/b protein complex, stress related Chl a/b binding protein) identified; Cr also induces modulation of proteins involved in amino acids metabolism.	Vannini et al. (2009)
Al	*G. max* (L.) Merr cvs. BaXi 10, BenDi 2 (root)	10% (w/v) TCA in acetone containing 0.07% (w/v) DTT, 1% PVP	7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (w/v) DTT, and 2% Pharmalyte pH 3–10	IPG, 2-DE, MALDI-TOF MS	30	Chaperones, PR 10, phytochrome B, GTP-binding protein, ABC transporter ATP-binding protein either newly induced or up-regulated, facilitate stress/defense, signal transduction, transport, protein folding, gene regulation, primary metabolisms.	Zhen et al. (2007)
	O. sativa L. cv. Xiangnuo 1 (XN1) (root)	40 mM Tris-base, 5 M urea, 2 M thiourea, 2% w/v CHAPS, 5% w/v PVP, and 50 mM DTT + ice-cold acetone with 0.07% (w/v) DTT	5 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer, 40 mM DTT	IPG, 2-DE, MALDI-TOF/TOF MS, MALDI-TOF-MS	17	Antioxidation and detoxification lead by up regulation of Al-responsive proteins (Cu–Zn SOD, GST, SAMS 2), ultimately related to sulfur metabolism. CS, a novel Al-induced protein, play key role in Al resistance.	Yang et al. (2007)

BABA, β-aminobutyric acid; CS, cysteine synthase; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Cp, Clipper; FBA II, fructose bisphosphate aldolase II; FBPase, fructose 1,6 bisphosphatase; GP, Golden Promise; IP, number of identified proteins; PPP, pentose phosphate pathway; Prx, peroxiredoxin; PGK, phosphoglycerate kinase; SAMS, S-adenosylmethionine synthetase; Sh, Sahara; TK, transketolase; Trx, thioredoxin; TBP, tributylphosphine; TEAB, triethylammonium bicarbonate; TSPP, tyrosine-specific protein phosphatase proteins; vBPO, vanadium-dependent bromoperoxidase.

COMPLEXATION, CHELATION, AND COMPARTMENTATION OF HMs WITHIN CELL

One of the important plant strategies of detoxifying HMs within cell is to synthesize low molecular weight chelators to minimize the binding of metal ions to functionally important proteins (Verbruggen et al., 2009). The thiol-containing chelating compounds strongly interact with HM, thus reducing free HM ions from cytosol and hence limiting HM toxicity (Cobbett and Goldsbrough, 2002). The phytochelatins (PCs) and metallothioneins (MTs), the two best characterized cysteine-rich HM binding protein molecules, play crucial roles in HM tolerance mechanism (Cobbett and Goldsbrough, 2002).

Phytochelatins synthesized from glutathione (GSH) by the enzyme PC synthase readily form complexes with HM in the cytosol and to facilitate their transport into vacuoles (Grill et al., 1989; **Figure 1**). Although PCs synthesis found to be induced in presence of most of the studied HMs, modulation of proteins, amino acids involved in PC biosynthesis have been the most widely studied in response to Cd. Our recent comparative proteome analysis of high and low Cd accumulating soybeans has revealed enhanced expression of glutamine synthetase (GS) under Cd stress. The enzyme GS is involved in the synthesis of GSH through glutamate biosynthesis pathway (Sarry et al., 2006; Semane et al., 2010). The enhanced expression of GS leads to more GSH formation (Hossain et al., 2012b). Induction of GSH synthesis implies higher metal binding capacity as well as enhanced cellular defense mechanism against oxidative stress (Verbruggen et al., 2009). Since GSH is the precursor of PC, enhanced expression of GS helps the cell to synthesize and accumulate more PC to detoxify cytosolic Cd^{2+}. Our finding is in agreement with previous reports of up-regulation of GS in response to Cd (Kieffer et al., 2008; Hradilova et al., 2010; Semane et al., 2010; Ahsan et al., 2012). In contrast, sharp decline in GS abundance has been reported in Cd-stressed rice roots (Lee et al., 2010).

Ahsan et al. (2012) exploited proteomic technique in combination of metabolomics for deeper understanding of PC-mediated detoxification of Cd^{2+} in soybean roots. Comparative analysis revealed that proteins (GS beta 1) and amino acids (glycine, serine, glutamic acid) associated with Cd chelating pathways are highly active in low root-to-shoot Cd translocating cultivar. In addition, proteins involved in lignin biosynthesis were shown to be increased under stress. Proteomic findings suggest that translocation of Cd ions from the root to the aerial parts might be prevented by the increased xylem lignifications.

The PC biosynthetic pathway has been finely dissected in Cd-exposed *Arabidopsis thaliana* cells using protein and metabolite profiling (Sarry et al., 2006). At high Cd concentration global pool of GSH decreased dramatically with the increase in dipeptide γGlu-Cys, suggesting high cellular demand of GSH for sustaining PC [$(\gamma Glu\text{-}Cys)_n$-Gly] synthesis.

Alvarez et al. (2009) implemented two quantitative proteomics approaches – 2-D DIGE and iTRAQ – to find out the relation between Cd^{2+} sequestration and thiol metabolism. Both techniques identified an increased abundance of proteins involved in sulfur metabolism. Sulfite reductase and *O*-acetylserine sulfhydrylase, involved in reduction of sulfate to cysteine, were found to be overexpressed in Cd-treated *Brassica juncea* roots. Authors suggested that under Cd-stress, sulfate availability for synthesis of PCs and GSH may limit Cd tolerance. Significant inductions of GSH and PCs (PC_3) in Cd-stressed rice roots further confirm the role of thiol-peptides in HM tolerance mechanism (Aina et al., 2007). Another proteomic study by Pandey et al. (2012) revealed higher abundance of cysteine synthase (CS) with higher contents of PCs and higher transcript of PC synthase in arsenic stressed *Anabaena* indicating their positive roles in As sequestration. Arsenic induced increases in GSH and PCs were also recorded in fronds of arsenic hyperaccumulator *Pteris vittata* (Bona et al., 2011). Interestingly, no such increase was evident in roots under As treatment. Proteomic results indicate that PCs could play role in As detoxification in *P. vittata* fronds only, but overall PC mediated detoxification is not the primary mechanism of As-tolerance in As hyperaccumulator, but to other adaptive mechanism. Up-regulation of proteins (CS and GSTs) and GSH pool involved in As detoxification has also been documented in proteomic study of As-stressed rice roots (Ahsan et al., 2008). Apart from Cd and As stress, CS and GSH also play essential role in Al adaptation for rice (Yang et al., 2007) and soybean (Zhen et al., 2007).

Unlike PCs, proteomics-based report on HM-induced alterations of MTs is very limited. Zhang et al. (2009) for the first time identified MT-like proteins from Cu-stressed germinating rice seed embryo. A number of gene expression studies have shown that MT genes are involved in Cu homeostasis and tolerance in *Arabidopsis* (Murphy and Taiz, 1995) and *Silene vulgaris* (van Hoof et al., 2001). Plant MTs not only play vital role in chelating Cu through the Cys thiol groups but are also considered as a potent scavenger of ROS (Cobbett and Goldsbrough, 2002; Wang et al., 2004).

The final step of HM detoxification involves sequestering of either free HMs or PCs-HMs complexes into cell vacuoles (Hall, 2002). This accumulation is mediated by tonoplast-bound cation/proton exchanger, P-type ATPase and ATP-dependent ABC transporter (Salt and Rauser, 1995; Hall, 2002). Transporters are also situated in plasma membrane and facilitate transport of HMs into apoplast. As the vacuoles or apoplasts have limited metabolic activity, accumulations of HMs in these compartments reduce the toxic effects of HMs (Schneider et al., 2009). The iTRAQ analysis of Cd-exposed barley leaf mesophyll tonoplast proteome led to the identification of ∼50 vacuolar transporters, that include vacuolar ATPase subunits, MRP-like ABC transporter and two novel CAX transporters (CAX1a and CAX5) and one Al-activated malate transporter protein (Schneider et al., 2009). Induction of these transporters especially cation/proton exchanger 1a and ABC transporter assure Cd^{2+} transport into vacuole (Aina et al., 2007). Further proteomic study by Lee et al. (2010) revealed induction of vacuolar proton-ATPase in rice roots and leaves indicating their positive role in Cd detoxification through vacuolarisation.

HM-INDUCED OXIDATIVE STRESS AND ALTERATION OF REDOX HOMEOSTASIS

Cellular ROS generation gets accelerated upon exposure to HM stress. HMs (Cu, Fe, Cr) that are directly involved in cellular redox reaction lead to ROS generation known as redox active, while redox inactive HMs (Cd, Al, As, Ni) trigger oxidative stress by depleting cells major thiol-containing antioxidants and enzymes, disrupting

electron transport chain or by inducing lipid peroxidation (Ercal et al., 2001; Hossain et al., 2012a). The excess intracellular ROS level alters protein structure by inducing oxidation of both protein backbone and amino acid side chain residues (Villiers et al., 2011). To counter stress, plants have evolved robust antioxidant defense mechanism comprised of both enzymatic and non-enzymatic components (Hossain et al., 2012d).

Most of the proteomic research done so far on HM-related toxicity revealed positive correlation between tolerance and increased abundance of scavenger proteins. Within plant cells, SOD constitutes the first line of defense against ROS. It plays pivotal role in cellular defense against oxidative stress, as its activity directly modulates the amount of $O_2^{\bullet-}$ and H_2O_2, the two important Haber–Weiss reaction substrates. The excess $O_2^{\bullet-}$ generated under HM-stress usually disproportionate into H_2O_2 by the action of SOD, which is then metabolized by the components of the ascorbate–GSH cycle. Higher expressions of SOD isoforms (Cu/Zn-SOD, Fe-SOD) have been documented in plants exposed to excess Cd (Kieffer et al., 2008, 2009; Alvarez et al., 2009; Farinati et al., 2009; Semane et al., 2010; Hossain et al., 2012b) and Al (Yang et al., 2007). Interestingly, root proteome analysis of Cd-exposed *B. juncea* revealed up-regulation of Fe-SOD while down regulation of Cu/Zn SOD (Alvarez et al., 2009). Ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) are involved in scavenging H_2O_2, hence protecting cell membrane from hydroxyl radical-induced lipid peroxidation (Barber and Thomas, 1978). The scavenging roles of APX, POD, and CAT have been documented in several proteomic studies related to Cd stress (Sarry et al., 2006; Aina et al., 2007; Kieffer et al., 2008; Lee et al., 2010; Hossain et al., 2012b) and As (Pandey et al., 2012) toxicity. Interestingly, excessive Cu (Bona et al., 2007), Cr (Sharmin et al., 2012) treatments or B deficiency (Alves et al., 2011) lead to decreased abundance of APX and POD. The detected suppression of POD is in accordance with the decrease in POD reported in maize roots treated with Al (Wang et al., 2011).

The abundance of another antioxidant enzyme of ascorbate–GSH cycle, monodehydroascorbate reductase (MDAR) was found to be increased in response to Cd (Sarry et al., 2006; Alvarez et al., 2009). MDAR helps to scavenge monodehydroascorbate radical and by doing this it generates dehydroascorbate (DHA), the oxidized form of ascorbate. Up-regulation of MDAR assures production of DHA, the substrate of dehydroascorbate reductase (DHAR) enzyme that catalyzes reduction of DHA to AsA (reduced ascorbate). In contrary, shoot proteome analysis of *Arabidopsis halleri* has shown decreased expression of MDAR in response to Cd, Zn, and rhizosphere microorganisms (Farinati et al., 2009). This down-regulation is also evident in roots of *Lupinus albus* undergoing long-term B deficiency (Alves et al., 2011). Decreased MDAR abundance in HM-stressed plants might indicate non-enzymatic disproportionation of monodehydroascorbate into AsA, essential for maintenance of balanced redox status (Hossain et al., 2009). Yet another well documented antioxidant found to be up-regulated under HM stress is peroxiredoxin (Prx). The Prx is basically a thiol peroxidase with multiple functions. It (a) detoxifies hydroperoxides; (b) plays essential role in enzyme activation and redox sensoring; (c) acts as molecular chaperone similar to HSPs; (d) induces cell signaling (Dietz, 2003; Dietz et al., 2006; Jang et al., 2004; Barranco-Medina et al., 2009). Prx was found to be induced under Cd (Sarry et al., 2006; Ahsan et al., 2007a; Hossain et al., 2012b) and As (Requejo and Tena, 2006; Pandey et al., 2012) stress.

Plants are also equipped with some additional defense proteins, shown to be up-regulated by HM stress. This group includes thioredoxin (Trx), Trx-dependent peroxidase, NADP(H)-oxido-reductase and glyoxylase I (Gly I). Trx is known to suppress apoptosis as well as supplies reducing equivalents to antioxidants (Hishiya et al., 2008). Excess Cu treatment seems to down-regulate the abundance of Trx and Trx-POD in germinating rice embryo (Zhang et al., 2009) and *Cannabis sativa* roots (Bona et al., 2007) respectively. However, enhanced expression of Trx was found to be helpful in mitigating oxidative stress in As-treated *Anabaena* (Pandey et al., 2012).

Methylglyoxal (MG), a cytotoxic by-product of glycolysis generally accumulated in cell in response to environmental stresses including HM (Espartero et al., 1995). MG readily interacts with nucleic acids and proteins causing alteration of function (Yadav et al., 2005). Detoxification of MG through glyoxalase pathway involves active participation of GSH and Gly I and Gly II enzymes. Up-regulation of Gly I was found to help the germinating rice seedlings in detoxifying MG under Cd (Ahsan et al., 2007a) and Cu (Ahsan et al., 2007b). Higher Gly I abundance was also reported in Cd + Zn + microorganisms treated *A. halleri* shoots (Farinati et al., 2009). Proteomic study also highlighted enhanced expression of NADP(H)-oxido-reductase by Cd (Sarry et al., 2006; Lee et al., 2010) and As (Pandey et al., 2012). This protein is a vital component of plants second line of defense, protecting cells from HM-induced oxidative damages.

Plants tolerance against HMs is often attributed to steady state of GSH pool for its multifunctional activities in PC synthesis, MG detoxification, ROS scavenging through ascorbate–GSH cycle, GSTs mediated decomposition of toxic compounds as well as stress signaling (**Figure 1**). Within GSH cycle, glutathione reductase (GR) acts as a rate limiting enzyme that catalyzes reduction of oxidized glutathione (GSSG) to GSH (reduced glutathione) and with the help of DHAR it maintains high AsA/DHA ratio necessary for tight control of HM-induced ROS scavenging. The delicate balance between GSH and GSSG is critical for keeping a favorable redox status for the detoxification of H_2O_2. Higher abundance of GSTs has been observed in response to Cd (Alvarez et al., 2009; Lee et al., 2010), As (Ahsan et al., 2008; Pandey et al., 2012), Cu (Zhang et al., 2009). Findings of Ahsan et al. (2008) revealed increased activity of GST-omega in rice roots following exposure of AsV, indicating the probable role of GST-omega in inorganic arsenic biotransformation and metabolism. The authors also suggested that depletion in GSH content may be associated with high rate of PCs synthesis thus detoxification of As through compartmentalization or due to down-regulation of enzymes of GSH biosynthetic pathways such as GR and CS. The HM-induced PCs synthesis coupled with GSH depletion is in agreement with earlier studies by Sarry et al. (2006) and Di Baccio et al. (2005).

Proteomic analyses strongly indicate that accumulation of defense proteins chiefly enzymatic components of ascorbate–GSH cycle, POD, CAT, GSH, GSTs, Gly I, Prx, Trx help cells to mitigate HM-induced oxidative stress by scavenging ROS.

MOLECULAR CHAPERONES

Protein dysfunction is an inevitable consequence of a wide range of adverse environmental conditions including HM toxicity. Molecular chaperones/heat-shock proteins (HSPs) are responsible for protein stabilization, proper folding, assembly, and translocation under both optimum and adverse growth conditions (Wang et al., 2004). In our study, enhanced abundance (>2-fold) of HSP70 protein was detected in leaves of high Cd-accumulating soybean cultivar Harosoy while low Cd-accumulating cv. Fukuyutaka exhibited decreased expression (Hossain et al., 2012b). Cd-induced up-regulation of HSP70 is also evident in response to various HMs including Cd (Kieffer et al., 2009; Hradilova et al., 2010; Rodríguez-Celma et al., 2010), Cr (Sharmin et al., 2012), and B deficiency (Alves et al., 2011). Ahsan et al. (2007a) reported increase of DnaK-type molecular chaperone BiP and chaperone protein HchA in germinating rice seedlings exposed to acute Cd toxicity. Al-stress also is known to induce one LMW-HSP and three DnaJ-like proteins in Al-stressed soybean (Zhen et al., 2007). To sum up, HSPs/chaperones play pivotal role in combating HM stress by re-establishing normal protein conformation and hence, cellular homeostasis.

HM-INDUCED ALTERATION OF PROTEINS INVOLVED IN PHOTOSYNTHESIS AND ENERGY METABOLISM

Down-regulation of photosynthetic machinery is a known phenomenon of Cd stress. Low abundance of proteins involved in photosynthetic electron transport chain and Calvin cycle has been reported in Cd-exposed *Populus* (Kieffer et al., 2008, 2009; Durand et al., 2010) and *Thlaspi* (Tuomainen et al., 2006). Pioneer proteomic work by Hajduch et al. (2001) of rice leaves exposed to HMs revealed drastic reduction in abundance/fragmentation of large and small subunits of RuBisCO (LSU and SSU), suggesting complete disruption of photosynthetic machinery by HM stress. This decrease in RuBisCO has also been documented in other HMs toxicity like As (Duquesnoy et al., 2009) and Cd (Kieffer et al., 2008). Proteomic analysis for other toxic HMs like As-exposed leaf proteome of *Agrostis tenuis* has shown total disruption of RuBisCO LSU and SSU along with oxygen-evolving enhancer protein 1 and oxygen evolving protein 2 in response to 134 μM As(V) treatment (Duquesnoy et al., 2009). Potassium dichromate treatment had similar effects on algal RuBisCO LSU and some antenna proteins namely light harvesting Chl a/b protein complex. However, Vannini et al. (2009) reported higher abundance of RuBisCO activase in *Pseudokirchneriella subcapitata* under chromate treatment. Interestingly, in our proteomic experiment with Cd-exposed soybean, increased abundance of RuBisCO LSU-binding protein subunits alpha and beta, RuBisCO activase, oxygen-evolving enhancer protein 1 and 2, NAD(P)H-dependent oxido-reductase, photosystem I and II-related proteins were evident (Hossain et al., 2012b). Enhanced expressions of proteins involved in photosystem I, II, and Calvin cycle might be an adaptive feature to overcome the Cd injury in soybean. This increased abundance is in accordance with the findings of Semane et al. (2010), who also reported increase of photosynthetic protein abundance in leaves of *Arabidopsis* treated with mild Cd stress. In our opinion, contribution of high photosynthetic assimilates into respiration would help plants to yield more energy needed to combat the Cd^{2+} stress.

To maintain the normal growth and development under stressed environment, plants need to up regulate metabolic pathways such as glycolysis and tricarboxylic acid (TCA) cycle. Detailed analysis of HM toxicity-related proteomic works has shown higher abundance of glycolytic enzymes phosphoglycerate mutase (PGM), glucose-6-phosphate isomerase (G6PI), triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), enolase (ENO), and pyruvate kinase (PK) in response to Cd (Sarry et al., 2006; Kieffer et al., 2008; Rodríguez-Celma et al., 2010; Hossain et al., 2012b), Cr (Labra et al., 2006). However, down-regulation of G3PDH was reported in As-treated rice roots (Ahsan et al., 2008) and roots of *Lupinus albus* under B deficiency (Alves et al., 2011). Similarly, Cu-treated *Cannabis* roots exhibited down-regulation of another glycolytic enzyme ENO, the metalloenzyme that catalyzes penultimate step of glycolysis – conversion of 2-phosphoglycerate to phosphoenolpyruvate (Bona et al., 2007).

Like glycolysis, enzymes of TCA cycle citrate synthase (CS), succinate dehydrogenase (SD), malate dehydrogenase (MDH), aconitase (ACO), aconitate hydratase (AH) were found to be up-regulated under Cd stress (Sarry et al., 2006; Kieffer et al., 2009; Rodríguez-Celma et al., 2010; Semane et al., 2010; Hossain et al., 2012b; **Figure 1**). In contrast, suppressions of several AH isoforms were evident in long-term B deficiency (Alves et al., 2011). Overall, up-regulation of glycolysis and TCA cycle might help the stressed plant to produce more reducing power to compensate high-energy demand of HM challenged cell.

ACCUMULATION OF PR PROTEINS IN RESPONSE TO HM STRESS

Plant cells trigger some common defense machineries whenever they encounter a biotic or abiotic stress. Accumulation of PR proteins is one of such plant defense strategies and often associated with systemic acquired resistance (SAR) against a wide range of pathogens (Van Loon, 1997; Durrant and Dong, 2004). Using the 2-DE approach, Elvira et al. (2008) successfully identified different PR protein isoforms (viz. PR-1, β-1,3-glucanases PR-2, chitinases PR-3, osmotin-like protein PR-5, peroxidases PR-9, germin-like protein PR-16, and NtPRp27-like protein PR-17) in *Capsicum chinense* leaves and additionally resolved their specific accumulation pattern in both the compatible and incompatible PMMoV–*C. chinense* interactions. Apart from the assigned role in plant defense against pathogenic constraints, PR proteins also play key role in adaptation to stressful environments including HM toxicity (Hensel et al., 1999; Rakwal et al., 1999; Van Loon and Van Strien, 1999; Hajduch et al., 2001; Akiyama et al., 2004; Edreva, 2005). Kieffer et al. (2008) documented marked increase in abundance of PR proteins class I chitinases (PR-3 family), several β-1,3-glucanases (PR-2 family), and thaumatin-like protein (PR-5 family) in Cd-exposed poplar leaves. Endo-1,3-beta-glucanase, a class 2 PR protein, also found to be induced in rice roots under short-term Cd stress (Lee et al., 2010). Higher abundance of PR proteins under HM as documented in many proteomic studies is in accordance with previous transcriptomic analysis of mercuric chloride-treated *Zea mays* leaves (Didierjean et al., 1996). Like Cd stress, PR-10 and LIR18B protein (both belong to PR-10 family),

and an acidic chitinase (PR-8 family) were *de novo* expressed under B deficiency (Alves et al., 2011). Stress-induced increase in ROS level has been shown to induce PR protein accumulation (Jwa et al., 2006). Treatment with excess Cu increased abundance of two PR proteins (PR-10a and putative PR proteins) in germinating rice embryos (Zhang et al., 2009). Analysis of the *Vigna unguiculata* leaf apoplast proteome using 2-DE and LC-MS/MS also revealed accumulation of several PR-like proteins glucanase, chitinase, and thaumatin-like proteins in response to excess Mn supply (Fecht-Christoffers et al., 2003). Transgenic tobacco overexpressing pepper gene CABPR1 encoding basic PR-1 protein showed enhanced resistance against HMs as well as pathogen stresses (Sarowar et al., 2005). These transgenic lines exhibited significant decline in total POD activity, suggesting that overexpression of CABPR1 in tobacco cells altered redox balance. Although, the precise role of PR proteins in combating HM stress is not yet clearly understood, the authors suggested that the induced redox imbalance might lead to H_2O_2 accumulation, triggering stress tolerance cascade. Several *in vitro* experiments have demonstrated that PR proteins display additional functions related to growth and development by modulating signal molecules (Kasprzewska, 2003; Liu and Ekramoddoullah, 2006). However, further proteomic investigations need to be undertaken to resolve the underlying molecular mechanism of PR proteins mediated plants HM tolerance.

CONCLUSION AND FUTURE PROSPECTS

The present review outlines the impact of HMs stresses on plant proteome constituents. Most of the investigations done so far primarily highlighted the differential expression of proteins involved in plant defense and detoxification pathways, namely ROS scavenging, chelation, compartmentalization. In addition, accumulation of PR proteins and modulation of plants vital metabolic pathways CO_2 assimilation, mitochondrial respiration in maintaining steady state of reducing power and energy required for combating HM-induced stress has been discussed in detail. Careful analysis of published proteomic works on HM toxicity has revealed that classical 2-DE coupled with MS-based protein identification has been the most widely used proteomic technique in investigating plant HM tolerance at organ/whole plant level. These proteomic findings have enriched us for deeper understanding plants HM tolerance mechanism.

The cellular mechanism of sensing stress and transduction of stress signals into the cell organelle represent the initial reaction of plant cells toward any kind of stress including HM. Communication through intracellular compartments plays a significant role in stress signal transduction process that finally activates defense gene cascade (Hossain et al., 2012d). To dissect the underlying molecular mechanism of how a plant cell modulates its protein signature to cope with stress, in depth study on organelle proteome would be of great contribution toward development of HM-tolerant crops.

As the PCs mediated HM-ion detoxification pathway ends in sequestration of PC-HM complexes into vacuole through various transporter proteins present in tonoplast membrane, more research on vacuole proteome needs to be undertaken for identification and characterization of novel metal transporter proteins responsible for cytoplasmic efflux of transition metal cations into vacuole. Legendary work by Schneider et al. (2009) on quantitative detection of changes in barley leaf mesophyll tonoplast proteome using advanced gel free iTRAQ method has enriched our knowledge about contribution of vacuolar transporters to Cd^{2+} detoxification. Plasma membrane proteome should be another target of future proteomic research on HM stress, as it acts as a primary interface between the cellular cytoplasm and the extracellular environment, thus playing a vital role in stress signal perception and transduction. Furthermore, transporter proteins present in cell membrane have importance in up-taking HM-ions into the cell. As most of the organelle membrane proteins are hydrophobic in nature, MS-based gel free system would be the most promising technique for identification of such proteins.

Plants response to multiple HMs would be another interesting area of future proteomic research (Sharma and Dietz, 2009). This could shed some light on cross talk between different HM stress signal pathways.

Heavy metal-induced protein oxidation study through redox proteomic approach has been the focus of much interest. More initiatives in this topic need to be taken as PTM/redox modification of proteins provides fundamental information about HM toxicity mechanism and biomarker discovery (Dowling and Sheehan, 2006; Braconi et al., 2011).

In summary, we believe that more research on sub-proteome-based HM approach would provide new insights into plants HM-stress response mechanism. HM-induced novel marker proteins would further enable us to design HM-tolerant transgenic crops.

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