REVIEW

Plasma membrane proteome in *Arabidopsis* and rice

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Plant cells contain many membrane systems that are specially adapted to perform particular functions. In plant cells, the processing of signals that are involved in responses to biotic and abiotic stressors occurs in the plasma membrane. Therefore, characterization of the plasma membrane proteome can provide new insights into the functions of various plant membrane systems. Plant plasma membrane proteomics can also provide valuable information for plant-specific biological investigations. Despite recent advances in preparative and analytical techniques for plant plasma membrane proteins, the characterization of these proteins, particularly the hydrophobic ones, remains challenging. In this review, plant plasma membrane proteomics data compiled from the literature on *Arabidopsis thaliana* are presented. Initial attempts to determine the physiological significance of some proteins identified from plasma membrane proteomics in rice and other plants are also described from the results of our research.

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1 Introduction

Plant cells contain many membrane systems that are specialized to perform particular functions. The plasma membrane, in particular, is an organized system that plays a structural role and functions as a communication interface with the extracellular environment for the exchange of information and substances. Environmental stresses, both biotic and abiotic, cause significant intracellular restructuring in plants [1]. In plant cells, the processing of signals involved in responses to biotic and abiotic stressors occurs in the plasma membrane. Therefore, a better understanding of the plasma membrane proteome would help in developing strategies to increase the natural defenses of plants. Indeed, in plant cells as well as in animal cells, the plasma membrane controls many primary cellular functions such as metabolite and ion transport, endocytosis, and cell differentiation and proliferation. In addition, the degree of association of proteins with the membrane varies, with some proteins being well embedded in the membrane lipid core, whereas other proteins are more peripherally located proteins that occasionally associate with the membrane through a reversible interaction [2]. Plant membrane proteomics can provide valuable information on plant-specific processes; however, the challenge for proteomics is to find methods of extracting and identifying the entire set of mainly hydrophobic plasma membrane proteins [3].

Proteome analyses at the level of subcellular structures require an analytical strategy that combines classical biochemical fractionation methods to enrich particular compartments with tools for the comprehensive identification of proteins. In plant cells and animal cells, one of the main potential benefits of this strategy is the capability of enhancing the understanding of the biochemical machinery of purified organelles for subsequent functional studies [4]. Proteomic analysis of membrane proteins remains a major challenge. Membrane proteins are more difficult to analyze than soluble proteins; moreover, the former are generally under-represented in datasets for several reasons. These are summarized by Ephritikhine *et al.* [5] as follows: (i) because of physicochemical heterogeneity, 2-D polyacrylamide gel electrophoresis (2-DE) separation is not appropriate for comprehensive mapping of membrane proteins; (ii) many hydrophobic proteins are not solubilized in the IEF sample buffer and precipitate at their isoelectric point; and (iii) low-abundance proteins, including rare membrane proteins, are beyond the LODs of standard proteomic techniques. The

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purity of the plasma membrane is also important for functional analysis. However, the plasma membrane in plant cells is difficult to isolate, possibly because of the presence of a rigid cell wall.

Analyses of multiple complete genome sequences of prokaryotes and eukaryotes have revealed that 20–30% of all genes encode transmembrane proteins. The complete sequencing of the genomes of *Arabidopsis thaliana* [6] and rice [7] enabled, for the first time, the systematic prediction of all putative plant membrane proteins. Recently, the annotation of the *A. thaliana* and rice genomes has progressed rapidly, and most of the predicted genes are now supported by full-length cDNAs [8–10]. Research on nonleguminous model species such as *A. thaliana* and rice provided useful insights into many fundamental aspects of plant biology. Legumes also are of immense importance to humanity and serve as a key element in sustainable agriculture. Two model species, namely, *Lotus japonicus* and *Medicago truncatula*, are the focus of genome sequencing and functional genomics studies [11]. Among those, it is not possible to identify plasma membrane proteins, because no signal peptide or specific signature specifying the targeting to plasma membrane, have been identified so far. In these cases, the proteomics approach can serve as a powerful tool for the analysis of the functions of plant macromolecules.

The advent of proteomics has enabled the identification of a broad spectrum of proteins in living systems. This capability is particularly useful for plants because it may provide clues, not only regarding their nutritional value, but also regarding their yield and the manner in which these factors are affected by adverse conditions. In this review, plant plasma membrane proteomics data compiled from the literature on *A. thaliana* are presented. Our initial research attempts to determine the physiological significance of some proteins identified from plasma membrane proteomics in rice and other plants such as soybean are also described.

2 Methodological tools for plasma membrane analysis in plants

To achieve all necessary cellular functions, plant cells require a large variety of proteins, including transport proteins, receptor proteins, and proteins involved in signaling or cellular traffic. For functional analysis, it is important to obtain plasma membranes in the pure state. Plasma membrane proteins are hydrophobic, lack solubilization properties, and are physicochemically heterogeneous so they cannot be separated by means of IEF. This hinders comparative analyses by 2-DE. Methods to isolate plasma membrane fractions vary according to organism, tissue, and cell types. Methods of isolation of plasma membrane fractions from a microsomal membrane fraction by two-phase partitioning from plant tissues are important and utilize the different surface properties of membranes. Plasma membrane proteins that do not span the lipid bilayer are generally well recovered after 2-DE. By contrast, the recovery of transmembrane proteins requires first the depletion of the plasma membrane fraction from soluble proteins by the use of specific solubilization procedures [12].

2.1 Plant plasma membrane protein extraction and solubilization for proteomic analysis

Proteomics analysis was used to describe the protein machinery of the plasma membrane of the model plant *A. thaliana*. This study revealed that the plasma membrane was rich in extrinsic proteins; however, the study encountered two major problems: (i) few hydrophobic proteins were recovered in 2-DE gels, and (ii) many plasma membrane proteins had no known function or were not included in the database, despite extensive sequencing of the *Arabidopsis* genome. Santoni *et al.* [13] reported that optimization of the solubilization procedures revealed that the choice of detergent to be used depends on the lipid content of the sample. The corresponding proteomes were compared by means of the statistical model of additive main effects with multiplicative interaction, which aims to regroup proteins according to their solubility and electrophoretic properties. Distinct groups emerged from this analysis and the identification of proteins in each group allowed the assignment of specific features to several of these proteins. For instance, two of these groups were composed of highly hydrophobic proteins; one group contained proteins with V-ATPase subunits, whereas the other contained proteins with one transmembrane domain and proteins known to interact with membrane proteins. This report will provide methodological tools for studying particular classes of plasma membrane proteins; such tools could also be applicable to other cellular membranes [13].

Sodium chloride and sodium carbonate, which have been proven to be efficient in previous analyses [14, 15], were chosen for use in plasma membrane proteome analysis. Marmagne *et al.* [16] were reported that a highly purified plasma membrane fraction was washed with solutions of NaCl and Na_2CO_3. Salt treatments of the membrane fraction with salts including NaCl and Na_2CO_3 are believed to abolish electrostatic interactions with integral membrane proteins or the polar head of lipids. Improperly or weakly linked membrane proteins are eliminated by such treatments, leading to enrichment in genuine membrane protein and to a lower sample complexity. Lefebvre *et al.* [17] isolated a root plasma membrane-enriched fraction from *Medicago* by phase partition and determined the lipid composition of detergent-resistant membranes compared with the root plasma membranes. Sphingolipids, free sterols, and steryl glycosides appeared as the main lipid components of these detergent-resistant membranes. A morphological comparison between plasma membranes and detergent-resistant membranes was also carried out by electronic microscope and it showed the high rigidity of detergent-resistant membranes and their strong ability to undergo self-adhesion.

Peskan *et al.* [18] reported for the first time the isolation of Triton X-100 insoluble fractions from tobacco membrane. Using tobacco, Mongrand *et al.* [19] also reported a detailed analysis of the lipidic composition of such a detergent-resistant fraction, indicating that it is highly enriched in a particular species of sphingolipid and in several phytosterols compared with the whole plasma membrane from originates. Bohnert *et al.* [20] isolated detergent-resistant membranes prepared from *A. thaliana* callus membranes. More *et al.* [21] first prepared a highly purified plasma membrane fraction from tobacco suspension cells, which constituted the first step in subcellular fractionation. This plasma membrane was further submitted to treatment with the nonionic detergent Triton X-100 to isolate the "detergent-resistant membranes; this should be considered as a further step in subfractionation. Because membrane proteins are poorly soluble in the detergents used for 2-DE, this limitation is all the more marked for proteins selected on the basis of their insolubility in nonionic detergent, the criterion for the isolation of detergent-resistant membranes. They have developed a buffer consisting of both ionic and nonionic detergents that are chaotropic to thoroughly solubilize detergent-resistant membranes. The subsequent problem was how to detect low-abundance proteins inside this complex mixture [21]. To investigate in that direction, an inventory of the proteins associated with these plant detergent-resistant domains seems essential.

2.2 Plant plasma membrane protein purification and solubilization using other approaches

Although much progress has been made recently on the study of proteomes, one of the current challenges remaining is the design of strategies for exhaustive quantitative proteomics to compare proteomes under various physiological conditions or from different genotypes. This has so far been mostly achieved by densitometric comparison of spot intensities after protein separation by 2-DE [3]. However, the physicochemical properties of membrane proteins preclude the use of 2-DE. Use of minor modification of isotope ratio to code sample for expression proteomics was investigated by Whitelegge *et al.* [22]. The $^{13}C/^{12}C$ isotope ratio of cells in culture was altered modestly manipulation of the source of CO_2 for photoautotrophic grown. Soluble proteins were separated from the membrane fraction after mechanical disruption of the cells. Membrane proteins were precipitated with acetone, dissolved in formic acid and subjected to analysis by LC-MS/MS.

Furthermore, Lanquar *et al.* [23] described the use of *in vivo* labeling by the stable isotope ^{15}N as an alternative approach for comparative plasma membrane proteomic studies in plant cells. *A. thaliana* cells were subcultured in medium containing either $K^{14}NO^3$ or $K^{15}NO^3$ as the sole nitrogen source. In this initial phase dedicated to the set up the experiment, total cell proteins were extracted from two cultures, quantified, and mixed at $^{15}N/^{14}N$ artificial ratios of 1:1 and 5:1. Quantification of ^{14}N *versus* ^{15}N MS signals reflects the relative abundance of ^{14}N and ^{15}N proteins in the sample analyzed. They described the use of ^{15}N-metabolic labeling to perform a partial comparative analysis of Arabidopsis cells following cadmium exposure. They could confidently identify plasma membrane proteins showing up to five-fold regulation compared to the unexposed cells. This study demonstrated that ^{15}N-metabolic labeling was a useful technique for comparative membrane proteome studies [23].

Furthermore, acoustic techniques can be used to homogenize protein pellets from various tissue samples of soybean or rice in a manner similar to the ordinary pestle-and-mortar method; the techniques give considerably better results than the vortex/ultrasonic method with respect to the resolution of the protein pattern through 2-DE. By using acoustic technology, noncontact tissue disruption, and protein pellet homogenization can be carried out in a computer-controlled manner that ultimately increases the efficiency of the process for a large number of samples. The introduction of a high-performance, single-tube sample preparation device (Covaris, Woburn, MA, USA) has enabled noncontact tissue disruption and protein pellet homogenization, thereby avoiding contamination and degradation [24]. Walsh-Haney and Coticone [24] previously used Covaris-Cryo-Prep and Covaris E200 for DNA extraction from human bones.

Toorchi *et al.* [25] used this system to extract proteins from the plasma membranes of soybean plants. For this technology, a lysis buffer containing tributylphospine, thiourea, and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate was preferred because protein quantification is possible by eliminating the interfering compound 2-mercaptoethanol and because of the high reproducibility of 2-DE separation. The results showed a comparable capability of acoustic technology to the mortar/pestle method, with regard to both rice and soybean, by providing reproducible and acceptable results [25]. This system yielded an excellent result for the 2-DE separation of plasma membrane proteins from soybean and rice tissues. At the laboratory scale, the mortar/pestle and water bath sonication methods are widely employed; however, both methods have their inherent limitations. A high-performance, single-tube sample preparation device for noncontact disruption, and uniform preparation of plant plasma membrane solubilization might provide new insights into the methods available at the laboratory scale.

3 Comprehensive proteomics analysis of plant plasma membrane

Many studies of plasma membrane proteomics have been conducted [2, 13, 26–28]. However, there are only a limited number of reports on the plasma membrane proteins of rice

[29, 30], because the plasma membrane in monocot plants is difficult to isolate. One of the reasons for this difficulty might be due to differences in cell wall polysaccharide composition between dicot and monocot plants. Membrane proteins are widely known as an unsuitable material for classical proteomic analysis using 2-DE followed by the identification of each spot by MS: many hydrophobic proteins are not solubilized in the IEF sample buffer and precipitate at their isoelectric point [13].

Previous 2-DE of tobacco leaf plasma membrane led to an estimation of 500 polypeptides [31], but with a clearly established under-representation of intrinsic membrane proteins. On the other hand, *A. thaliana* plasma membrane using MS directly after SDS-PAGE separation identified 97 hydrophobic plasma membrane proteins [2] and 238 putative plasma membrane proteins [28]. In the case of rice, the purified proteins of the plasma membrane were separated by SDS-PAGE or 2-DE and analyzed by LC-MS/MS. One hundred four proteins were detected, of which only 25 proteins were identified by 2-D PAGE. On the other hand, using SDS-PAGE and MS, 146 proteins were identified. Hence, the separation and identification powers of SDS-PAGE/MS were four times higher than those of 2-D PAGE/MS (Hashimoto, M., Toorchi, M., Matsushita, K., Iwasaki, Y., Komatsu, S., Proteome analysis of rice root plasma membrane and detection of cold stress responsive proteins, unpublished paper) (Table 1).

3.1 Comprehensive proteomics analysis of plant plasma membrane based on 2-DE mapping

In the study by Santoni *et al.* [32], no signal transduction and defense proteins were identified in the plasma membrane proteome of *A. thaliana*, although proteins involved in metabolism and energy were present [32]. A plasma membrane fraction purified from *A. thaliana* by using a standard procedure was analyzed by 2-DE. The proteins were classified according to their relative abundance in plasma membrane or cell membrane supernatant fractions. Eighty-two of the 700 spots detected on the plasma membrane 2-D gels were microsequenced and more than half of these spots showed sequence similarities to proteins of known function. Of these, all the spots in the plasma membrane-specific and plasma membrane-enriched fractions, together with half of the spots with similar abundance in the plasma membrane fraction and supernatant, had been previously found in the plasma membrane, thereby supporting the validity of this approach. They indicated that approximately 550 polypeptides found in the plasma membrane could be resolved on 2-D gels and numerous proteins with multiple locations were found in the plasma membrane. They also have shown that approximately 80% of the plasma membrane-specific spots correspond to proteins with unknown function, and half of these proteins with unknown functionality are represented by ESTs or cDNAs in databases. Although several unknown gene products were potentially localized to the plasma membrane, many of these proteins are now "known" with the completion of the genome sequence [6] and the great progress in annotation [8, 9]. These data were studied with respect to the efficiency of organelle proteome approaches for systematic linking of genomic data to genome expression and it was concluded that with the completion of *Arabidopsis* genome sequencing, generalized proteomes could serve as a powerful resource for genome-wide analyses of plant function.

In the study by Tanaka *et al.* [29], the plasma membrane proteins isolated from rice seedlings were separated by 2-DE and analyzed with Image Master 2D Elite software. The authors obtained 2-D maps of the 464 resolved proteins in the plasma membrane and analyzed the most abundant proteins on 2-DE by Edman sequencing and MS. Edman sequencing showed that the number of N-terminally blocked proteins varied widely among the subcellular compartments and that a much larger proportion of the proteins were N-terminally blocked in the plasma membrane (96%) than elsewhere. To restrict the possible role of the more abundant proteins in the subcellular membrane fractions of rice, the identified proteins were categorized according to criteria used by Bevan *et al.* [33]. It was found that in the plasma membrane of rice cells, proteins with functions associated with metabolism, energy, signal transduction, and defense were abundant [29].

Table 1. A summary of published papers on plant membrane proteomics

Plant	Methods	I/D	Identified proteins	References
Arabidopsis	2-DE, microsequence	82/700	Metabolism, energy functionally unknown	[32]
Rice	2-DE, microsequence, MALDI-TOF MS	90/464	Metabolism, energy, signal transduction, defense	[29]
Arabidopsis	SDS-PAGE, MALDI-TOF ESI MS/MS, LC-MS/MS	102/-	Transporter, functionally unknown	[2]
Arabidopsis	nano-LC-MS/MS	446/-	Receptor-like kinases, *etc.*	[16]
Arabidopsis	nano-LC-MS/MS	238/-	Transport, signal transduction, membrane trafficking, stress	[28]
M. truncatula	nano-LC-MS/MS	270/-	Receptor kinases, *etc.*	[17]

I/D, Number of identified proteins/Number of detected proteins.

3.2 Comprehensive proteomics analysis of plant plasma membrane based on SDS-PAGE/MS or LC-MS/MS

Marmagne *et al.* [2] attempted to identify new transport systems and ion channels. Although channel activities have been characterized in plasma membrane by electrophysiology, there have been few reports on the corresponding molecular entities. Indeed, the hydrophobic protein structure of plant plasma membrane remains largely unknown, although several proteomic approaches have been reported. Approximately 100 putative plasma membrane proteins were identified in hydrophobic protein-enriched plasma membrane fractions obtained from an *Arabidopsis* cell suspension culture. Ninety-five percent of these proteins are newly identified plasma membrane proteins and 50 of these proteins were predicted to contain transmembrane domains. Marmagne *et al.* [2] used 1-D gels for the analysis. To the best of our knowledge, no nonionic or zwitterionic detergent has been found to resolve plasma membrane proteins quantitatively from eukaryotic cells in the first dimension of 2-DE. Therefore, till date, it has not been possible to use 2-DE to resolve integral proteins of eukaryotic plasma membranes.

The proteomics of the plasma membrane has yielded sparse and partial information on the actual protein repertoire. Marmagne *et al.* [16] analyzed highly purified plasma membranes by means of nanoscale (nano) LC-MS/MS. With a total of 446 proteins identified, this was the most diverse plasma membrane proteome reported at that stage. Half of the proteins were predicted to contain transmembrane domains and to be anchored to the membrane, thereby validating *a posteriori* the pertinence of the approach. A detailed analysis highlighted two specific and novel features: the main functional category was represented by a majority of previously unreported signaling proteins as a receptor-like kinases, and a quarter of the identified proteins were predicted to be lipid modified, specifically involving double lipid linkage through N-terminal myristoylation, *S*-palmitoylation, C-terminal prenylation, or glycosyl phosphatidylinositol anchors. These results suggested that plasma membrane proteins were enriched in peripheral proteins transiently interacting with the plasma membrane [16].

Alexandersson *et al.* [28] analyzed highly purified *Arabidopsis* plasma membranes from leaves and petioles by nano-LC coupled online to an ESI MS for the identification of integral and peripheral proteins associated with the plasma membrane. A total of 238 putative plasma membrane proteins were identified, of which 114 were predicted to contain transmembrane domains or to be anchored by glycosyl phosphatidylinositol. Approximately two-thirds of the identified integral membrane proteins were actually not plasma membrane proteins. Of the 238 identified proteins, three-quarter of proteins were classified according to their function, and the major classes of proteins included transport, signal transduction, membrane trafficking, and stress responses. Almost a quarter of the proteins identified in this study have no known function, and more than half of these proteins were predicted to be integral membrane proteins [28]. Functional characterization of these unknown proteins may provide clues that could lead to the identification of novel functions for plant plasma membranes.

Several studies have provided new insights into the role of sphingolipid/sterol-rich domains the so-called lipid rafts of the plasma membrane from mammalian cells, and more recently from leaves, cell cultures, and seedlings of higher plants. Lefebvre *et al.* [17] showed that lipid raft domains, defined as Triton X-100-insoluble membranes, can also be prepared from *M. truncatula* root plasma membranes. These domains have been extensively characterized by ultrastructural studies as well as by the analysis of their content in lipids and proteins. *M. truncatula* lipid domains were shown not only to be enriched in sphingolipids and Delta[7]-sterols, with spinasterol as the major compound, but also in steryl glycosides and acyl-steryl glycosides. A total of 270 proteins were identified with nano-LC-MS/MS [17]. Among them, receptor kinases and proteins related to signaling, cellular trafficking, and cell wall functioning were well represented, whereas those involved in transport and metabolism were poorly represented. Evidence was also given for the presence of a complete plasma membrane redox system in the lipid rafts.

4 Functional analysis using plasma membrane proteome approaches

The proteins analyzed in the above-mentioned studies represent only a small part of the plant proteome and many other stress-responsive proteins remain to be identified. For example, the noncytosolic proteins, such as membrane proteins and nuclear proteins, are believed to play key roles in osmosensing and signal transduction. Although 2-DE has been widely used as a tool for proteomics, it has some limitations and technical difficulties, including the detection of abundant proteins. The depth of proteome coverage typically is low in 2-DE gels, with abundant proteins dominating the analysis. Proteins with high expression are visualized easily on stained 2-DE gels and usually mask low-abundance proteins, which may therefore need to be resolved by performing subcellular fractionation prior to 2-DE [34]. The publications on functional analysis using plasma membrane proteome approaches are summarized in Table 2.

4.1 Functional analysis in *Arabidopsis* using plasma membrane proteome approaches

Freezing stress injures the plasma membrane and induces the expression of stress-related proteins. Kawamura and Uemura [27] reported that in *Arabidopsis*, enhanced freezing tolerance was detectable after cold acclimation at 2°C for as short as 1 day and maximum freezing tolerance was attained

Table 2. A summary of published papers on plant membrane proteomics for functional analysis

Plant	Treatments	Methods	No.	Identified proteins	References
Arabidopsis	Cold acclimation	2-DE, SDS-PAGE, MALDI-TOF MS	38	Proteins associated with membrane repair, protection, CO_2 fixation	[27]
Arabidopsis	Brassinosteroid	2-D DIGE, LC-MS/MS	19 + 6	BAK1 receptor kinase, BZK1 transcription factor	[35]
Arabidopsis	Bacterial elicitor flagellin	iTRAQ, LC-MS/MS	8	RbohD, *etc.*	[36]
Rice	Salt	2-DE, MALDI-MS/MS	28	Plasma membrane pump/channels, membrane structure, oxidative stress, *etc.*	[30]
Alga	Salt	2-D blue native/SDS-PAGE, LC-MS/MS	20 + 2	Membrane structure, signal transduction	[39]

after 1 wk. To identify the plasma membrane proteins that change in quantity in response to cold acclimation, a highly purified plasma membrane fraction was isolated from leaves before and during cold acclimation, and the proteins in the fraction were separated by gel electrophoresis. They found that there were substantial changes in the protein profiles after as short as 1 day of cold acclimation. Subsequently, using MALDI-TOF MS, they identified 38 proteins that changed in quantity during cold acclimation. The proteins that changed in quantity during the first day of cold acclimation include those that are associated with membrane repair by membrane fusion, protection of the membrane against osmotic stress, enhancement of CO_2 fixation, and proteolysis.

In *Arabidopsis*, the plant hormone brassinosteroid binds to the extracellular domain of a receptor kinase and initiates a phosphorylation/dephosphorylation cascade that controls gene expression and plant growth. Tang *et al.* [35] detected early brassinosteroid signaling events and identified early response proteins using prefractionation and 2-D DIGE. Proteomic changes induced rapidly by brassinosteroid treatments were detected in phosphoprotein and plasma membrane fractions by 2-D DIGE but not in total protein extracts. Analysis of gel spots by LC-MS/MS identified 19 brassinosteroid-regulated plasma membrane proteins and 6 proteins from phosphoprotein fractions. These proteins include the BAK1 receptor kinase and BZR1 transcription factor of the brassinosteroid signaling pathway. Both proteins showed spot shifts consistent with brassinosteroid-regulated phosphorylation. In addition, *in vivo* phosphorylation sites were identified for BZR1, 2 tetratricopeptide repeat proteins, and a phosphoenolpyruvate carboxykinase. Overexpression of a novel brassinosteroid-induced plasma membrane protein partially suppressed the phenotypes of a brassinosteroid-deficient mutant, demonstrating its important role in brassinosteroid responses [35].

Advances in proteomics techniques have allowed the large-scale identification of phosphorylation sites in complex protein samples, but new biological insight requires an understanding of their *in vivo* dynamics. Nuhse *et al.* [36] demonstrated the use of a stable isotope-based quantitative approach for pathway discovery and structure–function studies in *Arabidopsis* cells treated with the bacterial elicitor flagellin. The quantitative comparison identified individual sites on plasma membrane proteins that undergo rapid phosphorylation or dephosphorylation. The data revealed both divergent dynamics of different sites within one protein and coordinated regulation of homologous sites in related proteins. They suggested that elicitor-responsive phosphorylation sites may reflect direct regulation of protein activity. These experiments demonstrated the potential for use of quantitative phosphoproteomics to determine regulatory mechanisms at the molecular level and provided new insights into innate immune responses [36].

4.2 Plasma membrane studies in the other species including rice

A key challenge is to identify the genes that confer salinity tolerance when plants are grown in different rice-growing environments and to use the identified genes efficiently to accelerate crop breeding [37]. A subcellular proteomics approach was used to monitor the changes in the abundance of plasma membrane-associated proteins in response to salinity. The proteins were extracted from a root plasma membrane-rich fraction of a salt-tolerant variety of rice, IR651, which was grown under saline and normal conditions. A comparative 2-DE revealed that 24 proteins were differentially expressed in response to salt stress, of which 8 proteins were identified by MS analysis. Most of the identified proteins were likely to be associated with the plasma membrane and were known to be involved in several important mechanisms of plant adaptation to salt stress. These mechanisms included regulation of plasma membrane pumps and channels, membrane structure, oxidative stress defense, signal transduction, protein folding, and the methyl cycle. To investigate the correlation between the mRNA and protein levels in response to salinity, they performed a quantitative real-time PCR analysis of three genes that were salt responsive at the protein level: 1,4-benzoquinone reduc-

tase, a putative remorin protein, and a hypersensitive-induced response protein. No correlation was detected between the changes in the levels of gene and protein expression. These results indicated that the proteomics approach was suitable for expression analysis of plasma membrane-associated proteins under salt stress [30].

The halotolerant alga *Dunaliella salina* uses a unique osmoregulatory mechanism and is able to proliferate in environments with extreme salt content [38]. The adaptation mechanisms involve major changes in the proteome composition associated with energy metabolism and carbon and ion acquisition. Katz *et al.* [39] performed a comprehensive proteomics analysis of the plasma membrane to clarify the molecular basis for the remarkable resistance to high salt. Plasma membrane proteins were recognized by tagging intact cells with a membrane-impermeable biotin derivative. Proteins were resolved by 2-D blue native/SDS-PAGE and identified by nano-LC-MS/MS. Their results showed that of 55 identified proteins, about 60% were integral membrane or membrane-associated proteins: novel surface coat proteins, lipid-metabolizing enzymes, a new family of membrane proteins of unknown function, ion transporters, small GTP-binding proteins, and heat-shock proteins. These datasets demonstrated that 20 protein spots increased and two protein spots decreased under high salt. The migration profiles of native protein complexes on blue native gels revealed oligomerization or comigration of major surface-exposed proteins, which may indicate mechanisms of stabilization in high salinity [39]. Thus, the assembly of protein interaction networks provides critical insight the inter-relationship of multiprotein complexes and the interconnections of their respective functions. Although a method of label-free quantitative proteomics developed by Sardiu *et al.* [40] is for human protein interaction networks, this method will become a useful tool for plant plasma membrane proteome.

Isobaric tags for relative quantification (iTRAQ) with LC-MS/MS might be useful for differential proteomics, because Nuhse *et al.* [36] have developed and applied to enrich for phosphopeptides from complex protein mixtures. So, there has been a trend that 2-DE gel-based approaches are replaced by LC-MS/MS-based profiling method. It is widely reported that LC-MS/MS has a better coverage of the proteome than 2-DE, because in theory some proteins such as hydrophobic membrane proteins cannot be separated in 2-DE while MS can detect any protein. In addition, Speers *et al.* [41] reported that elevated temperature was necessary and sufficient to maximize protein and peptide identifications for the shotgun analysis of membrane proteomes using standard C18 RP materials (Phenomenex, Torrance, CA, USA). Most μLC-MS/MS analyses are currently conducted at room temperature, the findings described herein should be of considerable value for improving the comprehensive study of integral membrane proteins. Chick *et al.* [42] demonstrated the use of peptide IEF on pH 3–10 IPG strips as the first dimension of 2-D shotgun proteomics for protein identification from the membrane fraction of mammalian cells. They reported that a comparison of different concentrations of methanol was assessed for assisting membrane protein digestion with trypsin prior to analysis using a gel-based shotgun proteomics approach, and identified a total of 800 proteins from 60% methanol. This technology will also contribute to Plant plasma membrane proteomics. However, until today, differential proteomics for identifying the functional proteins needs ability to detect and quantity change in proteins (Table 2). In this case, it is indicated that 2-DE and LC-MS/MS are highly complementary proteomic methods.

5 Conclusion

The proteomes of plant subcellular compartments can be fully described based on the wide variety of physiological and biochemical reactions occurring in different membrane systems. Numerous membrane-associated proteins in plants remain to be isolated and characterized [5]. To facilitate proteomics analyses, more powerful bioinformatics tools capable of analyzing large sets of proteins have been developed, including tools for systematically analyzing membrane protein structures [43]. On the other hand, there is a lack of accurate, reliable programs for functional annotation of proteomes, particularly in the case of rice membrane proteins. Application of a combination of proteomic strategies to membrane subproteomes may assist such functional annotation. By considering the dynamic nature of membrane proteomes resulting from the diversity between organs and organelles and the functional specificity of membrane systems, we predict that PTMs in response to stresses may help elucidate protein functions.

Analysis of membrane proteins remains a major challenge for proteomics techniques based on 2-DE, and so alternative methods based on either SDS-PAGE [44] or the separation of digested peptides have been described [45, 46]. Although these methods have been successful in identifying membrane proteins, they are inferior to a combination of quantitative analysis and degree of separation of protein variants by 2-DE. The solubilizing power of various nonionic and zwitterionic detergents as membrane protein solubilizers for 2-DE has been reported [47]. These results suggest that additional strategies must be used to clarify the characteristics of membrane proteins. Furthermore, many plasma membrane proteins are probably expressed only in certain types of cells at discrete development stages or in response to a particular stress. This dynamic nature implies that a large majority of plasma membrane proteins remain to be identified. In addition, the functions of many of these proteins are presently unknown; many proteins identified in the present study are functionally unclassified and more than half of them are predicted to be integral membrane proteins.

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