Mass Spectrometry Untangles Plant Membrane Protein Signaling Networks

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Plasma membranes (PMs) act as primary cellular checkpoints for sensing signals and controlling solute transport. Membrane proteins communicate with intracellular processes through protein interaction networks. Deciphering these signaling networks provides crucial information for elucidating in vivo cellular regulation. Large-scale proteomics enables system-wide characterization of the membrane proteome, identification of ligand–receptor pairs, and elucidation of signals originating at membranes. In this review we assess recent progress in the development of mass spectrometry (MS)-based proteomic pipelines for determining membrane signaling pathways. We focus in particular on current techniques for the analysis of membrane protein phosphorylation and interaction, and how these proteins may be connected to downstream changes in gene expression, metabolism, and physiology.

Simple Beginning: The Significance of PM Proteins
PMs play a crucial role in the cellular structure by providing a physical barrier between cells and their environment. They constitute a sensor for modifications to the cellular environment and a platform for intricate orchestration of signal transduction, allowing translation of external signals into finely tuned adaptive responses. Plant PMs are structurally highly complex and are very different from those of animals because they are surrounded by cell walls, and this diversity allows them to have distinct regions that can fulfill different roles, despite their fluid nature.

Membrane proteins that are integrated into – or attached to – the lipid bilayer are the main mediators of plant membrane functions. For instance, transmembrane proteins carry out selective transport of small molecules, thus establishing controlled exchange between the inside and the outside of the cell. Such transport processes are mediated by dozens of membrane-localized transporters [1,2]. Analyses of multiple genome sequences estimate that 20–30% of the cellular proteome consists of transmembrane proteins [1,3]. Membrane proteins are associated with PMs and other membrane-containing organelles such as plastids, chloroplasts and mitochondria. We focus here mainly on PM proteins not only because they represent a large group of plant membrane proteins, suggesting that they have special importance in plant signaling, but also because recent research has uncovered interesting new aspects of biological regulation.

Cellular sensing, signaling, and transporter regulation mediated through interactions with membrane proteins are of utmost importance to plants. Plant PMs contain many kinases, receptors, and enzymes, which are key regulators of these crucial cellular processes. Membrane-localized receptor-like kinases (RLKs) such as BRI1 and FLAGELLIN INSENSITIVE 2 function in signaling pathways [4,5]. Arabidopsis thaliana RLKs comprise a large family with >610 members – >75% of these contain transmembrane domains (TMDs). Several leucine-rich repeat (LRR)-RLKs have proven functional roles in regulating plant growth, morphogenesis, and responses to the environment [6–9]; however, the functions of most RLK family members remain unknown.

Highlights
Membrane receptors, kinases, and transporters communicate with intracellular processes through protein interaction networks. Characterization of plant PM proteins – especially hydrophobic proteins – remains challenging despite advances in separation and analysis techniques. Rapid advances in MS instrumentation and data analysis have enabled marked progress in deciphering the membrane proteome and mapping protein interaction partners, leading to a better understanding of PM protein complexes. Analysis of membrane protein phosphorylation under specific cellular conditions is crucial for elucidating the molecular mechanisms underlying signal sensing, transport, and metabolic processes. Phosphoproteomics allows unbiased localization and site-specific quantification of in vivo protein phosphorylation, thus facilitating the dissection of membrane signaling networks.

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Owing to the relatively low abundance of membrane proteins and the short timescale of these dynamic signaling events, it is notoriously challenging to analyze membrane protein function [10]. Although proteomic technologies for the analysis of soluble proteins (see Glossary) have progressed rapidly in recent years, membrane proteins have lagged behind and are typically under-represented in datasets. Gel-based proteomic approaches are limited in their ability to resolve basic or hydrophobic proteins as well as those with greater than three transmembrane regions. In addition, limited dynamic range and difficulty in identifying low-abundance proteins make lack of sensitivity a key issue.

With advances in high-throughput MS, shotgun proteomics and shotgun phosphoproteomics have increasingly become powerful alternatives to gels for analyzing complex protein samples [11–16]. Compared with intact protein analysis in top-down proteomics, peptides are easier to work with because they are readily solubilized and separated before MS, and are easily dissociated to produce useful fragmentation ions for identification. The breakthrough in efficient proteome-wide analysis of membrane proteins came with development of suitable membrane isolation methods, and extensive experimental studies of membrane proteomes have been reported in Homo sapiens, Mus musculus, and yeast [17–19]. In plants, large-scale analysis of several membrane proteomes is advancing [20–22] and detailed views of membrane phosphoproteomes have been generated [16,23,24]. These studies represent a fundamentally different approach to studying cell signaling. However, membrane proteomics remains a multifaceted, rapidly developing, and open-ended endeavor. Despite tremendous recent success, membrane proteomics still faces significant technical challenges.

In this review we focus on MS-based bottom-up proteomics methodologies for identifying plant membrane proteins, phosphorylation, and protein–protein interactions (Figure 1). Specifically, we briefly cover advances in MS for determining membrane phosphorylation and address the need for future experimental strategies to better understand membrane signaling networks on a proteome-wide scale.

**Challenges in the Preparation of Membrane Proteins**

Cellular membranes consist of a phospholipid bilayer and associated proteins which are categorized according to how they associate with the membrane: integral or intrinsic proteins traverse the membrane, whereas peripheral or extrinsic proteins are associated with components residing in the membrane but do not themselves reside within the membrane [25]. Integral membrane proteins are fundamental to many physiological processes and constitute 20–30% of the cellular proteome [26]. Most integral membrane proteins have TMDs; these proteins are often studied by MS because they act as cell-surface markers, receptors, or adhesion factors, and their cytoplasmic domains operate in cellular signaling pathways [27–29]. Some membrane-anchored proteins are tethered to the membrane bilayer by a lipid anchor. Such proteins have hydrophilic characteristics of soluble proteins and can be dissociated upon cleavage of the anchor using phospholipases [26].

Integral membrane proteins containing multiple TMDs tend to aggregate and precipitate upon removal from the lipid bilayer, and this appears to be exacerbated by the presence of reactive thiolates of reduced cysteinyl residues. Unfortunately, TMDs are poorly represented in bottom-up proteomics because their lack of charged residues leads to very poor peptide recovery; however, they can be accessed using top-down proteomics [30]. In addition, varying sizes of cytoplasmic and extracellular loops, as well as post-translational modifications (PTMs), confer diverse physicochemical properties to integral membrane proteins, resulting in sample losses with some sample preparation methods. Therefore, efficient extraction and digestion methods
MS to Determine the Complete Membrane Proteome

Several recent technological advances, including improved sample preparation, instrumentation, and better liquid chromatography (LC) performance, have led to a substantial increase in PM protein representation in large datasets [34,35]. However, membrane preparation and subsequent steps are decisive for improving membrane protein coverage. Purification of membrane proteins often involves cell lysis and sequential centrifugation steps to remove cell debris and isolate the membrane from the soluble fraction. Further purification can be accomplished by ultracentrifugation followed by density gradient centrifugation (e.g., using sucrose, sorbitol, or Percoll) [36] or filter-assisted digestion [37]. Accordingly, highly purified microsomes can be prepared by an aqueous–polymer two-phase partitioning step in which membrane capture is followed by 'shaving' (peptides are cleaved from the intact microsome surface) [16].

Typically, detergents are used to promote membrane protein solubility, for example a low concentration of SDS or Brij-58 is used to solubilize the membrane-enriched fraction. However, these reagents require multiple processing steps to remove them before LC/MS analysis, which can result in sample loss and quantitative variability. Alternatively, membrane proteins can also be solubilized using organic solvents in a methanol-bicarbonate buffer [27]. In Arabidopsis this approach resulted in a twofold increase in the detection of membrane proteins such as transporters and RLKs [27]. Although membrane proteins can be easily enriched by centrifugation, this lacks the resolution to provide highly purified PM fractions without substantial contamination by membranes from other subcellular organelles [38]. PMs share similar physiochemical properties with other membrane components and tend to exist as multiple structures. Fractionation procedures that reduce the protein complexity of the membrane proteome increase the chances of identifying proteins of low abundance.

To improve MS detection capabilities, isolated proteins or peptides can be fractionated using multidimensional chromatography, strong cation exchange (SCX) LC has proved to be effective in gel-free multidimensional analyses of complex peptide mixtures [19]. This step facilitates an increase in proteome coverage by partitioning peptides into fractions of reduced complexity that can be more comprehensively sampled by the mass spectrometer. The exploitation of more than one property of a peptide is a powerful chromatographic technique for protein identification from complex samples [27,39].

Optimization of peptide chromatography and processing can also facilitate analysis of low-abundance proteins in complex mixtures, for example by the use of very long monolithic columns.
with very high separation capacities [40]. This has helped in deep probing of the membrane proteome and represents an active area of research that holds great promise [19,41]. Inspection of the prefractionation screen chromatogram allows fine adjustment of the sample elution gradient and intelligent pooling of peptide fractions based on peak distributions and intensities [23].
A major advance in improving membrane proteome coverage is the use of less-hydrophobic resins, such as C4 columns instead of the commonly used C18 material, and this has been valuable for the separation of very hydrophobic peptides [42]. The application of heat to reverse-phase LC can also enhance the identification of extremely hydrophobic proteins and peptides [43]. In addition to improving the performance of affinity chromatography, use of alternative enzymes to trypsin or Lys-C for protein digestion as well as modifications to the digestion protocol can increase the number of peptides available for MS analysis [44,45].

Membrane proteins are highly dynamic and vary between different cell types. Comparison of the proteome with transcriptome profiles is a good way to determine whether a MS analysis is comprehensive. Although transcript and protein levels might not correspond entirely, they should provide a useful qualitative benchmark of the portion of the protein-encoding genome that could potentially be identified in an MS experiment [46]. For accurate peptide identification, the highest-scoring peptide spectrum match (PSM) is taken as a candidate for the identity of the peptide. Most workflows control the false discovery rate using a target decoy approach [47]. Fragmentation spectra are searched not only against the target database but also against a decoy database designed to produce false positive PSMs.

**MS-based Proteomics to Analyze Membrane Microdomains**

MS is also effective for analyzing proteins of **membrane microdomains** named lipid rafts, which represent a very active area of membrane research. These specialized membrane domains have been studied by preparing the **detergent-resistant membrane (DRM) fraction** from purified PM fractions [48]. However, plant DRMs have been understudied relative to their animal counterparts. Proteomic analysis in several species has demonstrated that such DRMs contain 5–10% of total PM proteins in plants [49–51]. All these proteome data point to the importance of membrane microdomain proteins and their dynamics during various signaling processes. These investigations suggest that the DRM protein profile is distinct from the whole PM proteome. However, many proteins that are clearly not related to the PM or its microdomains copurify with DRMs. One way to tackle this problem is through quantitative proteomics in combination with complementary biochemical approaches. Quantitative methods including label-free quantification, stable isotope labeling, and isotope dilution have been applied to DRM proteome analysis, showing that many DRM proteins localize to distinct microdomains of plant cells [52,53]. Microdomain-specific localization of DRM proteins functions in the establishment of cell polarity, membrane trafficking, and specialization of host membranes upon interaction with microbes [54].

Current proteomic technology allows identification of >1000 DRM proteins and the relationship with their localization in specific membrane microdomains [53]. Other evidence from quantitative proteomic analysis indicates that rapid and discrete modifications of proteins associated with DRMs take place upon stimulation with pathogen-associated molecular patterns [55]. Consistent proteomic data were obtained for *Arabidopsis* cells following flg22 stimulation, tobacco cells treated with the elicitor cryptogein [56], and rice challenged with chitin for 5–15 min [57]. The similarity of the timing of such modifications (5–15 min) deserves a mention, as does the fact that different complementary approaches further confirm the involvement of most identified DRM proteins in the early steps of immune signaling as well as their association with PM microdomains [4,58,59]. These quantitative proteomic analyses have revealed a restricted list of proteins that are specifically enriched in DRMs that define specialized functional structures.

**MS-based Methods to Determine Membrane Protein Interaction Networks**

Systematic large-scale analyses of protein–protein interactions among membrane proteins are clearly under-represented despite the utmost importance of membrane protein complexes in
transport processes as well as in signal perception and transduction. In-depth characterization of individual PM protein complexes in plants suggests a highly modular and dynamic system in which protein–protein interactions are adjusted according to cellular conditions [60].

MS is one of the most powerful tools for identifying and quantifying proteins, determining PTMs, and for exploring protein–protein interactions (Figure 2A,B). Plant membrane protein interactions can be complex and are a crucial determinant of function that can be effectively investigated using MS. Analysis of protein complexes through immunopurification (IP) followed by MS is widely used because of its high throughput and sensitivity. Suitable controls and quantitation are required for distinguishing bona fide binding proteins from background contaminants.

Figure 2. Large-Scale Mass Spectrometry (MS)-Based Approaches for Unraveling Phosphorylation-Driven Membrane Signaling Networks. (A) Targeted or untargeted interactome strategy for unraveling protein interaction partners. Protein interaction partners are isolated using one-step or tandem tag affinity purification, the eluate is subjected to MS for proteomic analysis. (B) Global membrane phosphoproteome strategy for discovering putative substrates of protein kinases and phosphatases. Experimental data ultimately need to be combined with systems biology analysis which translates the separate, large-scale datasets into signaling networks. Predicted connections within and between signaling cascades need to be experimentally verified by, for instance, analysis of protein complexes and analysis of kinase or substrate mutant plants. Abbreviation: LC-MS, liquid chromatography with mass spectrometry.
IP-MS has recently been applied in plant aquaporin interactome studies [61], enabling the detection of more than pairwise interactions, although the technique is limited by the number of bait proteins tested. Owing to the high dynamic range, membrane protein–protein interaction is difficult to study using traditional methods such as IP using specific antibodies that recognize the bait proteins. Moreover, specific antibodies are often not available; in contrast to the animal and human fields, plant protein antibodies are relatively scarce, hindering high-throughput analysis of the plant protein interactome.

Recent affinity purification MS (AP-MS) methods have used epitope tags on target proteins directly in the genome as affinity capture probes for identifying interacting proteins, without requiring specific antibodies for each new bait protein (Figure 2A) [62]. The principle of AP-MS is to isolate the bait from the plant sample by affinity purification under near-physiological conditions; binding partners of the bait can then be recovered from the sample and identified by MS. One key attribute of MS-based approaches for such analysis is that they can potentially be applied to any membrane protein in vivo given that this technique requires no specific antibodies. Despite the high numbers of membrane protein interaction partners identified using a split-ubiquitin interaction screen [1], this method investigates interactions ectopically in yeast, whereas AP-MS is closer to the endogenous system of plants. The resulting datasets also differ from those of other approaches that deliver pairwise interactions, and AP-MS yields co-complex data (both direct and indirect interactions). Therefore, different techniques tend to deliver complementary datasets.

Initial studies of membrane protein interactions by MS were small in scale and focused on well-characterized proteins of interest, such as analysis of the Arabidopsis cell-cycle interactome [63]. This is because the short, single-step purification used in AP-MS experiments leaves behind considerable amounts of background contaminants. Compared with single-step methods, tandem affinity purification (TAP) detects membrane protein–protein interactions with a higher signal-to-noise ratio and also allows purification of protein complexes [64]. The TAP tag originally developed in yeast was improved for plants, yielding the TAPi tag [62]. This was further boosted through implementation of the GS tag (a fusion between protein G and streptavidin-binding peptide) which is superior to the original TAP tag in both specificity and complex yield [65]. Applying this method to Arabidopsis cells, Pauwels and coauthors revealed that TOPLESS-related proteins affect multiple jasmonate signaling pathways through interaction with specific adaptor proteins [66].

A prior fractionation step for baits present in specific subcellular fractions can increase membrane protein interactome coverage [67]. The development of easy purification protocols and ultra-sensitive MS has allowed widespread use of TAP-MS for screening the membrane interactome of plants. Moreover, studying protein complexes in their developmental context through the isolation of PMs has now become feasible. TAP has been successfully adopted for screening membrane partners of the circadian multiprotein complex and the protein kinase interactome [68–72]. These investigations have uncovered novel signaling events involved in the interaction networks of target proteins such as cyclin-dependent kinase, phytochrome, and target of rapamycin (TOR) kinase. Another interactomic analysis using affinity enrichment effectively identified interactive proteins of membrane-bound receptor complexes such as LRR kinases [73]. The two-step TAP system reduces background contaminants and identifies stable interactions, but unlike single-step AP often does not retain transient interactions. These approaches are complementary to genetic methods such as yeast two-hybrid screens in which proteins are overexpressed and must be able to interact in the yeast nucleus. Combined with quantitative approaches, TAP-MS can
identify condition-specific protein interactions and also allows a dynamic view of the membrane protein interactome.

Quantitative approaches permit the use of mild IP protocols and allow specific membrane-binding proteins to stand out by their quantitative signature, even among a very large background of proteins. Using high mass accuracy MS, a newly reported integrative analysis combining phosphoproteomics and interactomics has identified dozens of novel signaling components of TOR kinase networks [70]. Overall, these MS-based approaches could prove valuable for kinase interaction network studies given that they enable the proteome-wide discovery of both up- and downstream network components. Because relying on the identification of proteins by pull-down leads to many false positives, it is crucial to distinguish background binders from significantly enriched bona fide interactors. Statistical tests can identify true interactors but require a control for comparison [74,75]. Owing to its quantitative nature, AP-MS can probe not only steady-state interactions but also dynamic rewiring upon stimulation by internal or external stimuli. In addition, because this approach isolates and enriches proteins, it can identify PTMs, either with or without further PTM enrichment, enabling in-depth assessment of the role of these modifications in regulating interactions. An AP-MS analysis of Arabidopsis ubiquitylation enabled direct detection of 950 ubiquitylated proteins, >100 of which had previously escaped detection [76]. In this regard, MS has the unique ability to identify and functionally profile previously unknown interactions from a complex biological state.

Cytoscape has emerged as the de facto standard for general-purpose network analysis [77]. Through its plugin infrastructure such as Stich, it provides a wealth of analysis and visualization tools, often integrating quantitative proteomic technologies with interaction networks. Independently of the biological question, the true value of MS in analyzing membrane protein interactions lies in its complementarity with biochemistry, molecular biology, and optical approaches. Quantitative MS approaches and continuous developments in bioinformatics can provide a precise definition of temporal changes in membrane protein interactions and are expected to continue expanding the range of AP-MS applications to plant biology studies.

Proteomics to Understand Protein Phosphorylation Signaling Networks Across Plasma Membranes

Techniques to Improve Phosphoprotein Identification

Protein phosphorylation is one of the most important PTMs in plant cells, and is involved in many biological functions including signal transduction, differentiation, hormone perception, transformation, and metabolism. All these signal transductions lead from receptor proteins at the PM to transcription factor proteins in the nucleus (Figure 3). More than 5.5% of the Arabidopsis genome comprises genes encoding kinases, and more than half of these gene products are located at membranes, suggesting a complex phosphorylation signaling network in plants [1]. The regulation of membrane proteins through phosphorylation has been studied extensively using purified proteins and by site-directed mutagenesis of target phosphorylation sites.

MS analysis of membrane protein signaling has gained attention in recent years and is revealing signaling pathways at unprecedented levels [20,28,78]. Membrane phosphoprotein analysis by MS is nevertheless more challenging because modified peptides may be present at low abundance, and the modification may be labile during fragmentation. In the context of signal transduction, this challenge is exacerbated by the generally low copy number of many proteins with pivotal roles in signaling cascades [1]. Fortunately, low abundance can often be alleviated by phosphopeptide purification – this purification captures all modified peptides of interest and no others (Figure 2B) [15,79–83]. In practice, phosphopeptides can be enriched more than
100-fold by resins that chemically coordinate the phosphogroups, for example by the use of metal oxide coordinators such as alumina and titanium dioxide [82–84]. The term **metal oxide affinity chromatography** (MOAC) was coined by us [83]. Furthermore, combined subsequent enrichment of phosphorylated proteins and their corresponding phosphorylated tryptic peptides — **tandem MOAC** — increases the coverage of low-abundance phosphoproteins [15,80,81,85]. Novel signaling targets for mitogen-activated protein (MAP) kinase and SnRK1-TOR kinase were successfully detected using tandem MOAC [15,81]. In plant membrane phosphoproteomics, MOAC has been successfully applied to phosphopeptide enrichment, yielding thousands of phosphoproteins [16,23,86]. However, generating large-scale datasets for membrane phosphoproteins usually requires fractionation of samples from a larger amount of plant material. A major breakthrough in the detection of phosphorylated membrane proteins was recently reported from *Arabidopsis* roots, where >2000 phosphorylation sites were identified from 1 g of plant material using a one-step membrane fraction enrichment strategy [87]. This method is promising for the identification of high-quality membrane phosphopeptides in large-scale proteomics and is applicable to low amounts of plant materials.
Quantitative Phosphoproteomics

Global membrane phosphoproteomic analysis follows the same generic shotgun proteomics workflow as phosphorylation-specific analyses, with an enrichment step inserted either at the membrane protein level or more typically at the peptide level. Even for phosphopeptide-enriched samples, confident identification of phosphopeptides and unambiguous localization of phosphorylation sites is more difficult than the identification of unmodified peptides because search engines need to consider additional possibilities. Rigorously determined false discovery rates for phosphorylation determination are important for accurate confirmation, especially when phosphorylation studies are intended to serve as community resources [88,89]. High mass accuracy in fragmentation spectra is required for unambiguous localization of phosphorylation sites, and this increases the confidence of both peptide identification and phosphorylation site localization. However, the goal of phosphorylation studies is usually not only to catalog many sites but also to determine how they change during cell signaling. This necessitates quantitative mapping or possibly time-resolved studies of phosphoproteomics [6,16,23,90].

To investigate dynamic phosphorylation signaling events in early plant hormone stimulation, we combined MOAC [83] with a novel data processing and mining technique called mass accuracy precursor alignment (MAPA) [91], which enables the alignment of MS peaks without dependence on database identification, and we applied this to the analysis of membrane phosphoproteins involved in phytohormone signaling [16,91]. In addition to label-free quantitative approaches, stable isotope labeling such as 15N-metabolic labeling-based phosphoproteomics allows accurate quantification of transient phosphorylation changes of membrane proteins [23]. These approaches coupled with genetic analysis were used to reveal several kinase–substrate networks involved in ethylene signaling relay pathways [92,93]. Using a similar strategy, Wu and colleagues characterized the in vivo function of a transmembrane sucrose-induced receptor kinase (SIRK1) in the context of sucrose resupply signaling in Arabidopsis seedlings [94]. In combination with single ion-reactions monitoring (SRM) on a triplequad mass spectrometer, target sites for specific phosphoproteins could be efficiently analyzed across a wide range of samples [95].

A very important and currently underestimated problem is to reference changes in quantitative protein phosphorylation measured by phosphoproteomic techniques to quantitative changes in the corresponding unphosphorylated proteins. This can be achieved by comparing phosphoproteomic and proteomic data from the same sample, as in our recent global quantitative phosphoproteomic studies [15,96,97]. These investigations indicate that quantitative membrane phosphoproteomics has enabled a deeper characterization of protein kinase signaling networks.

In contrast to the large number of kinases, the Arabidopsis genome encodes about 150 protein phosphatases [98]. A previous phosphoproteomic study revealed that BRASSINOSTEROID SIGNALING KINASE 8 is involved in the regulation of SUCROSE PHOSPHATE SYNTHASE (SPS) through activation of a phosphatase, which in turn dephosphorylates and thus activates SPS [78]. A more recent phosphoproteomic analysis using a PROTEIN PHOSPHATASE 2C (PP2C) mutant found an enrichment of several kinase phosphorylation motifs [99]. These reports suggest that phosphatases control phosphorylation sites by directly or indirectly modulating the activities of enzymes or kinases. Overall, quantitative membrane phosphoproteome analysis provides an informative perspective on cell signaling and will be crucial for modeling signaling networks in a systems biology approach.

Functional Phosphoproteomics

Mutational analysis of protein kinases or specific phosphorylation sites will provide clues about their involvement in plant signaling. However, a key attraction of MS is that it can quantify kinase-associated changes in the entire signaling network. Thus, quantitative phosphoproteomics can be applied to the functional analysis of many membrane (or membrane-associated) protein...
kinases, such as BRI, BAK1, abscisic acid (ABA) receptors (PYR1/PYLs), CIPKs, SnRKs, and RLKs (Figure 3) [6,15,28,100]. In combination with genetic analysis, phosphoproteomic studies have revealed several substrates of SnRK2 kinase by quantifying phosphorylation changes between *Arabidopsis snrk* mutants and wild-type materials [101,102]. A recent functional phosphoproteome study reported that sucrose stimulus activates SIRK1, and that SIRK1 interacts with and phosphorylates another coreceptor membrane kinase named QSK1 to regulate the phosphorylation state of aquaporins [29].

Identification of a range of possible substrates has provided insight into the role of protein kinases; however, more mechanistic approaches are now being adopted that attempt to integrate all observed changes rather than merely focusing on the most dramatic or most reproducible. High-quality phosphorylation datasets containing thousands of quantified phosphorylation sites are an attractive resource for system-wide analysis of signaling networks (Figure 3). For example, recent analysis of TOR kinase phosphorylation provided evidence that network modeling can identify crucial phosphorylation sites that are key control points for membrane-localized ABA receptors [28]. Similar studies also demonstrated that MAPKs activated by nutrient limitation phosphorylate PM channel boron transporters, which in turn control the polarization of boron transport in *Arabidopsis* roots [80]. Integration of information about cell type-specific kinase expression, cellular localization, substrate phosphorylation motifs, and other functional interaction data with global phosphorylation data is useful for predicting kinase–substrate relationships in signaling networks (Figure 3) [6,96,97,103].

Connecting kinase motif and phosphorylation-dependent binding domains with *in vivo* phosphorylation data further helps in matching phosphorylation sites to their putative up- or downstream kinases, and computational analysis of phosphoproteomic data can be used to investigate the molecular evolution of signaling networks [79,86]. In addition to finding partners for full-length proteins, membrane proteomics is uniquely suited for exploring phosphorylation-mediated interactions in signaling networks. The power of quantitative proteomics to discover phosphorylation-induced protein interactions was recently demonstrated in an example inspired by interactomic analysis of TOR kinase signaling networks [70]. Proteomics and interactomics reveal complementary subspaces of kinase signaling pathways, enabling system-wide discovery of network components for deciphering cellular membrane signaling networks (Figure 2A,B).

Phosphoproteomics methods are becoming the methods of choice for decoding membrane protein functions at a system level. As shown here, membrane phosphoproteomics is now used for mapping signaling networks in considerable depth, for delineating cellular control points, and, owing to its unbiased nature, for uncovering unexpected biological connections. However, future progress in MS-based membrane proteomics and phosphoproteomics necessitates further significant improvements in instrumentation before we can comprehensively and routinely analyze whole membrane proteomes or phosphoproteomes. Furthermore, increasing the dynamic range of phosphoproteomics will be necessary to determine substoichiometric phosphorylation sites on low-abundance membrane proteins in the presence of high-abundance phosphopeptides.

**Other PTMs**

Although MS can in principle analyze all PTMs, phosphorylation is by far the most extensively studied PTM of membrane proteins; hence we focus here on explaining methodologies and key applications of phosphoproteomics. Other PTMs such as nitrosylation and glycosylation are also important, and a range of approaches have been developed to characterize them using MS; however, high-throughput analysis is only beginning. Previous glycoproteomic analysis
using lectin enrichment of N-linked peptides yielded 318 N-glycosylation sites in proteins extracted from tomato fruits, but there was less enrichment in membrane proteins [104]. Efforts directed at O-linked complex glycans are gaining ground. The development of bioinformatics tools has generated databases of plant membrane proteins and their PTMs that are accessible on the Internet [105,106]. These can be useful for identifying networks of proteins that are highly conserved among species and for suggesting possible roles for unknown proteins. However, in many cases these data are predicted by programs or come from in vitro experiments, which often generate false positive identification rates. In addition, subcellular predictions occasionally fail because some proteins do not have an exclusive destination but are dually targeted between cellular organelles [107]. Therefore, no attempt has been made to extrapolate ‘consensus’ targeting information. Experimental data are presently the only reliable information on protein targeting to subcellular compartments. Stringent parameter setting and manual validation of MS spectra are required for accurate PTM confirmation. Overall, although the analysis of phosphorylation by MS is relatively straightforward, comprehensive determination of all PTMs remains a daunting endeavor.

**Concluding Remarks**

Membrane networks receive and transmit signals that manifest as decisions to modulate physiological functions in response to environmental changes. The complexity of these signaling networks requires the use of high-throughput proteomic approaches to investigate how information is processed and how protein–interactor relationships are determined. In recent years, MS has evolved from producing mere lists of identified membrane proteins to producing highly sophisticated strategies that are indispensable for the analysis of complex biological systems. These unexpected findings have had a significant effect on the membrane protein field, propelling it in new and exciting directions. As shown here, MS can now be used to comprehensively identify membrane proteins, map membrane protein–protein interactions, and elucidate membrane signaling networks in considerable depth (see Outstanding Questions). Considering the crucial role played by membrane proteins in cellular function and signaling interactions, membrane proteomics is likely to play a major and indispensable role in the future study of signaling modulation.

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**Outstanding Questions**

How can we increase membrane proteome or phosphoproteome coverage for MS analysis?

How can we determine if MS analysis of a given sample is truly comprehensive?

How can we control for false positives during peptide identification? What are the advantages of gel-free proteomics relative to gel-based techniques?

Can the TAP-MS approach be applied to any plant species and any biological state interactions?

What determines the probability of PTMs during the identification of protein phosphorylation sites?

Can protein interaction partners be screened using an MS method?

How do membrane protein kinases modulate intercellular signaling networks?

Can the molecular function of an individual phosphorylation site be characterized using MS methods?

Can networks of kinases and their substrates be deciphered at a proteome-wide level?

What defines the resolution and precision of large-scale phosphorylation site mapping?
37. Wensel, J. et al. (2009) Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. J. Proteome Res. 8, 5974–5979
46. Schmollinger, S. et al. (2014) Nitrogen-sparring mechanisms in Chlamydomonas affect the transcriptome, the proteome, and photosynthetic metabolism. Plant Cell 26, 1410–1435
53. Gutierrez-Carronel, E. et al. (2016) A shotgun proteomic approach reveals that fs deficiency causes marked changes in the protein profiles of plasma membrane and detergent-resistant microdomain preparations from Beta vulgaris roots. J. Proteome Res. 15, 2510–2524
59. Lu, X. et al. (2017) Membrane microdomains and the cytoskeleton constrain AHNR1 dynamics and facilitate the formation of an AHRT-associated immune complex. Plant J. 90, 2–16