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Review Article

Mapping the plant proteome: tools for surveying coordinating pathways

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Plants rapidly respond to environmental fluctuations through coordinated, multi-scalar regulation, enabling complex reactions despite their inherently sessile nature. In particular, protein post-translational signaling and protein–protein interactions combine to manipulate cellular responses and regulate plant homeostasis with precise temporal and spatial control. Understanding these proteomic networks are essential to addressing ongoing global crises, including those of food security, rising global temperatures, and the need for renewable materials and fuels. Technological advances in mass spectrometry-based proteomics are enabling investigations of unprecedented depth, and are increasingly being optimized for and applied to plant systems. This review highlights recent advances in plant proteomics, with an emphasis on spatially and temporally resolved analysis of post-translational modifications and protein interactions. It also details the necessity for generation of a comprehensive plant cell atlas while highlighting recent accomplishments within the field.

Introduction

Since the early 2000s, mass spectrometry-based proteomics has undergone rapid evolution. Increased ionization and ion transfer efficiencies, fragmentation options, and resolving power have made mass spectrometers more powerful than ever before [1–9]. Coupled with enhanced high-resolution liquid-based separation techniques, protein identifications, dynamic range, and reproducibility have markedly improved [10–14]. Biological investigations with increased depth and breadth have revealed protein networks as well as unveiled the exquisitely complex circuity of regulation via post-translational modifications (PTMs) [15–18]. Method development advances continuously expand the biological relevance and necessity of proteomic analysis in the understanding of metabolic functions; while the genome is static, the proteome rapidly responds to external stimuli to regulate the epigenome, transcriptome, and metabolome to maintain cellular homeostasis [19–23]. In plants, this is even more essential, as their sessile nature mandates rapid, multiscalar responses to survive fluctuating environments [24–30]. While identification, quantification, and characterization of proteomic responses allows for robust, hypothesis driven studies of fundamental processes in plant biology (recently reviewed: [31–36]), improvements in temporal and spatial specificity must be leveraged to capture the dynamic landscape of the plant proteome [31, 37–39].

Proteomics is essential for the understanding of major biochemical signaling pathways involved in plant adaptations, including those affected by biotic and abiotic stressors, requisite symbiosis, climate change, and optimization for desired characteristics (e.g. biofuels, agriculture, etc.) [36, 40–42]. Although mRNA abundance is often used as a proxy to understand lifespan and turnover of proteins, only modest correlation between mRNA expression levels and protein levels has been demonstrated [37, 43–46]. RuBisCo, the most abundant protein on earth, exemplifies this discrepancy in correlation, with 10–100× greater protein expression levels than would be predicted from transcript abundance [47]. Additionally, while transcriptomes respond to stressors/stimuli, the proteome generates metabolic responses and shifts the metabolome through protein folding, turnover, enzymatic activity, and

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substrate specificity via coordinated modulation of protein abundance, PTMs, protein–protein interactions, and conformational changes [48–55]. Thus, proteome-level investigations are required to understand the spectrum of dynamic intracellular metabolic states [50, 56–64].

Despite these advances, plant proteomics has historically lagged behind its mammalian counterpart. Although plant science is crucial to address critical global concerns, research funding for plant science has long trailed that of biomedical research and innovative bioanalytical techniques are likewise hyper focused on mammalian disease models [65]. This too has extended to proteomics; for instance, in the most well characterized model plant species, *Arabidopsis thaliana,* it is estimated that only 5% of its proteome has been experimentally characterized to distinguish function, localization, and biological significance [47, 66, 67]. In addition to funding disparities, plants incur unique challenges relative to mammalian systems including a recalcitrant cell wall that hinders protein extraction without intense denaturing methods [35, 68]. Plants also have low cytoplasmic volume relative to cell mass as well as high protease and phosphatase abundance, making it more challenging to accurately assess the proteome without artifactual modifications caused by the stress of cell lysis and sample processing [69–71]. Additionally, plant proteomes vary in dynamic range over 6–8 orders of magnitude, precluding detection of low abundance proteins in shotgun proteomic approaches [72]. This is particularly influenced by the highly abundant photosynthetic apparatus, as >20% of expressed protein in photosynthetically active plant tissue is derived from the chloroplast alone [62, 73]. Furthermore, despite the high biodiversity of plants (>300 000 species of land plants, alone), genome sequencing in plants lags behind its mammalian counterparts; with less than 600 full genome sequences available, proteomic investigations across the plant kingdom are inherently limited [74]. Despite these inherent challenges, comprehensive understanding of plant pathways is necessary to address the most pressing issues of modern times, including food security, rising global temperatures, and the need for renewable plastics and fuel sources. As such, the plant science community has called for the generation of a 'plant cell atlas,' through which multiple lines of data can be integrated to extensively profile plants on the basis of species and cell type [75]. This review outlines the progress and barriers to comprehensively mapping the plant proteome.

Advances in post-translational modifications

Quantitative mass spectrometry-based proteomics has revolutionized our understanding of PTMs and their functions across the plant proteome [76–79]. While the low ratio of modified to unmodified proteoforms prevents thorough examination via a shotgun approach, enrichment strategies have been optimized to allow site-specific identification and quantification of a diverse repertoire of modifications in plants, including those for phosphorylation, oxidation, acetylation, and ubiquitination [80]. Furthermore, characterization of PTMs in isolated cell types as well as organelles has provided increased depth of coverage and a broad framework for investigating the relationships between PTMs and cell/organelle function [81–85]. When combined with shotgun approaches, enrichment techniques enable unparalleled discovery and accurate quantification of PTM events.

Phosphorylation and Cys oxidation: star-crossed modifications

The most well-characterized plant PTM is phosphorylation, due in part to the disproportionately high level of kinases encoded by plant genomes; approximately 5% of the *Arabidopsis* genome encodes protein kinases, nearly double that of mammals [86]. These critical genomic differences in highly conserved eukaryotic pathways yield significant alterations in intracellular regulation that delineate the need for robust proteome analysis across the plant kingdom. Discovery-based proteomics has thus revealed divergent post-translational signaling networks across phosphorylation pathways, including those in the highly conserved target of rapamycin (TOR) and mitogen-activated protein kinase (MAPK) regulatory networks, indicating the presence of novel regulatory proteins and functions not present in the animal kingdom (Table 1) [87, 88]. Still yet, functional determination of phosphorylation sites is complicated by the abundance of phosphorylated proteins paired with the low stoichiometric ratio of phosphorylated to non-phosphorylated residues, as well as the spatial and temporal specificity of signaling networks. Recent work employed immobilized metal affinity capture (IMAC) to quantify 43 000 phosphosites in *Arabidopsis*, with phosphorylation present on 47% of the proteome [73]. Furthermore, by separating the plants into 30 distinct tissue types, it was revealed that phosphorylation forms distinct tissue-dependent patterns throughout the plant, further supporting the need for comprehensive, tissue-specific delineation of PTM signaling networks. Studies have also shown differential phosphorylation is regulated by the circadian rhythm, with protein phosphosites involved in photosynthesis, translation, metabolism and cellular transport changing in abundance based on the plant's diurnal cycle [89, 90]. Further studies could build upon

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Table 1 Online repositories containing data on post-translational modifications of various plant species

Repository	PTMs	Plant Species	Website
dbSNO	*S*-nitrosylation	*Arabidopsis thaliana*	http://dbsno.mbc.nctu.edu.tw/
Functional Analysis Tools for Post-Translational Modifications	Acylation, Lys-acetylation, N-glycosylation, O-GlcNAc, Phosphorylation, *S*-nitrosylation, SUMOylation, Ubiquitination	*Arabidopsis thaliana*	https://bioinformatics.cse.unr.edu/fat-ptm/
PhosPhAt 4.0	Phosphorylation	*Arabidopsis thaliana*	http://phosphat.uni-hohenheim.de/
Plant Protein Phosphorylation database	Phosphorylation	*Arabidopsis thaliana* *Brassica napus* *Glycine max* *Medicago truncatula* *Nicotiana tabacum* *Oryza sativa* Solanim tuberosum *Vitis vinifera* *Zea mays*	http://www.p3db.org
Plant Proteome DataBase	Amino acid substitution, Deamidation, Hydroxylation, N-terminal acetylation, N-terminal formylation, Oxidation, Phosphorylation, Propionylation	*Arabidopsis thaliana* *Zea mays*	http://ppdb.tc.cornell.edu/
Plant PTM viewer	Carbonylation, Lys-2-hydroxyisobutyrylation, Lys-acetylation, Lys-malonylation, Lys-methylation, Lys-succinylation, Lys-SUMOylation, Lys-ubiquitination, N-glycosylation, N-terminal acetylation, N-terminal myristoylation, N-terminus proteolysis, O-GlcNac, Oxidation, Phosphorylation, *S*-glutathionylation, *S*-nitrosylation, Ubiquitination	*Arabidopsis thaliana* *Chlamydomonas reinhardtii* *Oryza sativa* *Triticum aestivum* *Zea mays*	http://www.psb.ugent.be/PlantPTMViewer
PTMcode 2	Acetylation, Carboxylation, Hydroxylation, N-glycosylation, Methylation, O-GlcNac, O-GalNAc, Parlmitoylation, Phosphorylation, *S*-nitrosylation, Ubiquitination	*Arabidopsis thaliana*	https://ptmcode.embl.de/
The Ubiquitination Site tool	Ubiquitination	*Arabidopsis thaliana*	http://bioinformatics.psb.ugent.be/webtools/ubiquitin_viewer/

established workflows for spatial proteomic analysis by combining with temporally resolved studies for tissue-specific analysis of the diurnal phosphoproteome.

While mammalian cells primarily generate ROS in the mitochondria, photosynthetic eukaryotes generate ROS from both the mitochondria and the chloroplasts [28]. The mitochondria, chloroplasts, and (to a lesser extent) the peroxisomes combine to form an intricately coordinated regulatory system that uses ROS for rapid intracellular signaling. As such, oxidative modifications have been increasingly scrutinized as advances in redox proteomics have unveiled reversible oxidative signaling to have a substantial role in all areas of plant metabolism including photoregulation, effector-triggered immunity, and nutritional sensing and regulation [57, 91–93]. Oxidative signaling networks cross-talk with phosphorylation, SUMOylation, and other PTMs to comprehensively regulate plant metabolism [28, 61, 83, 94–96]. However, the analysis of redox-modified proteoforms is

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limited due to challenges in sample preparation and enrichment, both of which can generate artifactual oxidation. Further, oxidative modifications, particularly those of cysteine thiols, incorporate diverse chemical groups and instigate varying functions, and it is currently not possible to both assess for the overall oxidation state while simultaneously differentiating between the diverse repertoire of oxidized modifications (e.g. glutathionylation, *S*-nitrosylation, disulfide bonds, sulfonic acid, etc.). Like phosphorylation, the low abundance of oxidized proteoforms (methionine and cysteine make up only 4.3% of all amino acids in *Arabidopsis*, combined), often necessitates the use of enrichment strategies for meaningful analysis. Furthermore, the occupancy of oxidative modifications occurs on a spectrum, introducing heterogeneity into data analysis that is challenging to overcome [97, 98].

Technological advances for distinguishing PTM-specific proteomes

Traditionally, phosphoproteomic studies have focused on phosphohydroxyamino acids formed from serine, threonine, and tyrosine residues. Commonly used enrichment strategies include immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), and polymer-based metal ion affinity capture (PolyMAC), all of which selectively bind the negatively charged phosphate groups under acidic conditions (Figure 1). Material selection in each method has a marked impact on the peptides enriched, with different methods showing preferential binding of mono- or multi- phosphorylated peptides, increased/decreased affinity for acidic residues, and other biases for the analysis of serine, threonine, and tyrosine residues. However, phosphorylation also occurs on six other amino acids, including histidine, lysine, arginine, cysteine, and aspartic and glutamic acid, all of which are prone to hydrolysis under acidic conditions that prevents traditional phosphoproteomic analytical methods [99]. Additionally, the polyphosphorylation of serine and lysine, known as pyrophosphorylation, adds further analytical complexity to an already elaborate network [100, 101]. Recent work has sought to improve phosphopeptide enrichment of non-hydroxyamino acids through the synthesis of novel affinity matrices and chemical probes, with improved detection through electron transfer/higher energy collisional dissociation fragmentation [102–105]. Additionally, strong anion exchange is a promising strategy for non-biased phosphopeptide enrichment, revealing 1/3 of the total basal human phosphoproteome to occur outside of hydroxyamino acids [106]. Although the field of labile phosphoproteomics is still in its infancy, non-canonical phosphorylation has already been implicated in the regulation of chlorophyll biosynthesis and cytokinin signaling, demonstrating that these modifications are likely implicated in essential plant pathways [107, 108]. Extending the technological advances used in mammalian systems has the potential to unveil critical and biologically significant phosphorylation networks across plant taxons.

Detection of oxidized cysteine thiols can be divided into direct and indirect approaches [109] (Figure 2). Indirect approaches block free sulfhydryl groups (i.e. thiols reduced *in vivo*) during protein extraction using an alkylating agent, primarily via the irreversible addition of iodoacetamide (IAM) or *N*-ethylmaleimide (NEM) [110]. Following the blocking of free thiols, reversibly oxidized cysteines are then nonspecifically reduced via a strong reductant (e.g. DTT, TCEP), or selectively reduced for the enrichment of particular modifications, such as the use of ascorbate to selectively reduce *S*-nitrosylation [97, 111–117]. The use of a strong reductant, while lacking modification specificity, enables the simultaneous probing of the full reversibly oxidized redoxome, unveiling critical oxidation targets that could be otherwise overlooked [56, 57, 61, 91, 118]. Recent work has progressively increased the strength of the reducing agent and labeled with isobaric tags to multiplex for the simultaneous analysis of distinct modifications [119, 120]. The development of isobaric thiol tags has also enabled high-throughput site-specific quantification of redox occupancy, allowing for robust quantification of heterogeneously oxidized proteoforms [121]. Further still, the use of concentration-dependent reactive probes following blocking and reduction allows for the determination of relative reactivity of cysteine thiols, with reactivity correlating with relative biological importance. Quantitative thiol reactivity profiling, while a recent technique, has already been successfully implemented in *Arabidopsis* and *Chlamydomonas reinhardtii* [122, 123].

Direct approaches for analyzing cysteine oxidation require fewer sample preparation steps and thus are less likely to generate artifactual oxidation; however, modification-specific chemical probes require a distinct labeling reagent for each oxidized derivative (e.g. *S*-sulfonation, *S*-nitrosylation, *S*-glutathionylation, etc.) [124–126]. Novel probes have enabled direct quantification of diverse redox modifications, including sulfonic acids, disulfide bonds, and nitrosylation [124, 125, 127, 128]. Most probes use an acetyl moiety connected to a preferentially binding functional group, such as the benzothiazine-based probe used to map over 1500 *S*-sulfenylation sites on over 1000 proteins in *Arabidopsis* [127]. Recent work has also transformed *Arabidopsis* with a tagged

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Figure 1. Overview of common mass spectrometry-based strategies for the analysis of canonical and non-canonical phosphorylation.

proteinaceous probe that specifically reacts with sulfenic acids and traps them through an irreversible mixed disulfide bond [93]. This *in vivo* trapping unveiled sulfenylated cysteines in over 1000 proteins, 45% of which had not been previously identified in the *Arabidopsis* redoxome, while preventing the problematic oxidation that occurs *ex vivo*. However, innate challenges in the genetic transformation of plants will prevent widespread use outside of a few model species. Furthermore, the use of a proteinaceous tag is analogous to crosslinking mass spectrometry and therefore increases the difficulty in identifying peptides (see section below).

PTM analysis is challenging due to their sub-stochiometric abundance, labile nature, and the tendency of artifactual modifications. While sample preparation methods can successfully enrich for modified proteins/peptides with great success, there are still challenges in data processing that prevent thorough analysis of PTM cascades. Fragmentation advances enable thorough mapping of peptide/protein sequences, yet specific fragments to localize PTMs are not guaranteed. Scoring algorithms embedded in database searching software somewhat

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Figure 2. Representative mass spectrometry-based strategies for the direct and indirect analysis of reversible cysteine oxidation.
Both approaches can be facilitated using peptide-level enrichment as well; however, recent work has favored protein-level enrichment due to the decrease in artifactual oxidation.

address the limitations [129–131], but in most situations users are required to pre-select expected variable modifications. As the number of possible variable modifications increases, the search space increases exponentially, thereby increasing both search time and the probability of false matches. Recent advances in tools for open modification searches has sought to decrease search time while increasing identification of modifications [132–137].

Plant interactomes reveal functional significance

While the identification and stoichiometry of proteoforms is crucial to understanding protein signaling, the latter is achieved through protein interactions and spatial specificity. To this end, the last decade has benefited from improvements in sample preparation techniques enabling subcellular localization of the chloroplast, mitochondria, and nuclear proteome, among others [58, 60, 138–142]. Additionally, mass spectrometry is employed to enhance the understanding of interactions that reveal the importance of intracellular spatial orientation of protein networks [143–146] (Figure 3). Protein–protein interactions inform the functions of genes and

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Figure 3. Common strategies for mass spectrometry-based interactomics that have been employed in plant systems.
Many of the approaches can be applied together for complementary analysis of protein networks.

individual proteins and provide critical analysis of cellular processes [147]. While over 48 000 interactions have been characterized in *Arabidopsis*, the wide range of plant effectors and subsequent metabolic responses is still largely uncharacterized [148]. However, interactomic techniques are challenging in plant systems due to their diverse metabolic pathways, complex and/or poorly annotated/uncharacterized genomes (particularly in non-model species), and the reliance of plants on symbiotic interactions with outside effectors [149]. Additionally, interactomic methods have historically utilized genetic manipulation, which can be challenging in plant systems, particularly among polyploids [150]. Increased implementation of differential interactomics is essential for the generation of a comprehensive plant cell atlas.

Chromatography-based interactomics

Genetic and/or antibody approaches for interactomics (such as AP-MS) rely on the sequence availability of the target organism and/or genetic transformation, both of which face inherent challenges in plant systems due to

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low transformation efficiency, lack of model organisms, and polyploidy. Size-exclusion chromatography (SEC) circumvents these obstacles to allow for non-biased separation of protein complexes in both sequenced and non-sequenced organisms. While SEC was originally paired with gel electrophoresis to allow for global analysis of the interactome in *Arabidopsis*, blue-native polyacrylamide electrophoresis (BN-PAGE) uses one dimensional fractionation to create size-distribution profiles in a single step, decreasing experimental complexity while still enabling characterization of >3000 proteins in a single experiment [151–153]. When paired with other organelle fractionation (often achieved through differential separation and Percoll gradients), it is possible to analyze low abundance complexes with both spatial specificity and unparalleled depth of coverage [152, 154–157]. However, this approach is challenging to use for quantitative analysis, in part due to proteins being present in an average of 17 fractions. To remedy this, clear-native (CN) PAGE uses customized R scripts to deconvolute elution peaks, resulting in 74% of identified proteins existing in one single fraction and increasing the probability of correct partner assignments [158].

Similar to PAGE-based complex separations, cofractional (CF) MS uses non-denaturing SEC to identify physical associations between proteins [159]. While this has been highly successful in characterizing protein complexes in *Arabidopsis*, false negatives can arise from coincidental co-elution in small experiments [160–163]. However, by using repeated coelution across multiple, distinct separations, the statistical power is increased, generating lower false-discovery rates and increasing the utility. Recent work employed CF-MS to characterize protein complexes across 13 plant species spanning the evolution of Viridiplantae, from single-celled *Chlamydomonas reinhardtii* to the vascular broccoli and *Arabidopsis* [47]. This work revealed, not surprisingly, that highly conserved eukaryotic protein functions are carried out in plant complexes containing divergent proteins compared to the mammalian counterparts, further indicating the necessity of enhanced plant proteomics. However, this work stopped shy of tissue-specific differential interactomics, the next step in generating a thoroughly characterized plant cell atlas. The identified interactomes could be used for a recently developed semi-targeted complex-centric approach, wherein data-independent acquisition (DIA) is used for precise differential quantification with temporal and spatial specificity [164].

Affinity purification mass spectrometry

Affinity purification combined with mass spectrometry (AP-MS) is a powerful and unbiased technique through which the soluble protein–protein interactions of plants have been extensively characterized [165]. AP-MS has been traditionally used to reveal components of protein complexes, such as the subunits of the evening complex, an essential complex for circadian regulation, in *Arabidopsis* [165–167]. However AP-MS can also identify transient binding partners, such as the relationship between cytosine methyltransferases and superoxide dismutases in moss, a discovery which revealed a previously unknown cross-regulatory role for methylation in redox homeostasis [168]. This is improved through computational tools that increase confidence in identifications [169–173]. However, while AP-MS has facilitated the characterization of protein complexes and binding partners from plants, it is less robust than in mammalian cell systems that benefit from the extensive availability of commercial antibodies. Although transgenic affinity tags have been used to a large degree of success, these can potentially change binding sites on proteins or change expression levels within the biological system [174–179]. Further still, both false positives and false negatives are common in AP-MS, requiring robust statistical testing and well characterized positive and negative controls to effectively discern positive identifications [180, 181]. Plants, particularly non-model species, are therefore more challenging to probe. Recent work has combined AP-MS with label-free quantitative proteomics to resolve the dynamic nature of the strigolactone pathway in *Arabidopsis*, demonstrating its potential for temporal resolution of interchanging protein partners [182]. AP-MS will therefore likely continue to be play a prominent role in determining plant protein interactions, something that will be expanded as further plant species are sequenced.

Proximity-dependent labeling

Proximity-dependent labeling (PL) employs engineered enzymes, usually ligases or peroxidases, to generate reactive radicals to covalently tag neighboring proteins with enrichable sidechains [144, 146, 183–186]. PL was first demonstrated *in planta* in *Arabidopsis* using BirA*, a mutated bioengineered biotin ligase derived from *Escherichia coli*, for proximity-dependent biotin identification (BioID) of interaction networks, where it enabled facile identification of 500 interacting proteins *in planta* [187]. Similarly, TurboID uses the same biotin ligase as BioID but has enhanced activity, through which it can label an equivalent amount of diverse enzymes in <1% of the time it takes BioID [188]. In a combined study of *Arabidopsis* and *Nicotiana benthamiana*, BioID

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revealed low abundant protein networks in both species as well as the nuclear proteome of stomatal guard cells [189]. Furthermore, the combination of TurboID and cell-specific enrichment was highly successful, enabling the differentiation of 34 previously non-localized guard-cell specific nuclear proteins and providing a critical framework for future differentially resolved spatial interactomic studies [190, 191]. While only benchmarked in plants in 2019, TurboID has uncovered the putative E3 ubiquitin ligase responsible for regulating the nucleotide-binding leucine-rich repeat immune receptor following exposure to *Tobacco mosaic virus* (TMV) as well as over 300 targets of the GSK3-like kinase, BIN2 [192, 193]. Its utility in determining TMV targets demonstrates TurboID's capacity to play a key role in differentiating the defense and symbiotic response networks established by plant pathogens, a critical area of research [27, 194, 195].

Crosslinking mass spectrometry

Crosslinking mass spectrometry (XL-MS) uses hetero- or homo-bifunctional tags to covalently modify amino acids, linking them to other residues within the defined distance of the spacer arm [196–198]. These highly reactive tags are only confined by the specificity for the functional group, enabling non-biased global analysis of both protein interactions as well as the spatial delineation of complexed proteins [199, 200]. Advancements in XL-MS have substantially accelerated in the last five years [201]. This has been assisted by the advent of MS-cleavable crosslinkers, some of which are also enrichable, as well as the ability to use MS^3 workflows to increase confidence in crosslinked identifications through the use of reporter ions [202–207]. Despite these new crosslinkers, data analysis remains one of the most significant challenges of XL-MS, as the linking of two peptides increases the potential search space by n^2. Over 20 algorithms have been developed for analyzing XL-MS data, with varying expertise needed for usage and interpretation (reviewed: [207]).

In order to discern the spatial proximity of proteins *in vivo*, the chemical crosslinker must have high membrane permeability. While XL-MS has been employed extensively in mammalian systems, its application to plants is complicated by the recalcitrant cell wall and has been limited. However, recent increases in the chemical repertoire has enabled investigations *in planta*, with highly permeable azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide crosslinkers leading the charge [208, 209]. Quantitative *in planta* XL-MS analysis of the *Arabidopsis* proteome was achieved through the use of azide-tag modified disuccinimidyl pimelate (AMDSP), a biotin-enrichable tag that facilitated the identification of 354 unique crosslinked peptides [209]. Further still, *in vitro* crosslinking was recently combined with phosphoproteomic analysis to spatially resolve 244 substrates of the *Arabidopsis* TOR pathway [210]. Integration of XL-MS with PTM analysis is an exciting step toward mapping essential signaling networks of plant systems.

Toward a spatially and temporally resolved future

Plant proteomics is an essential line of evidence in the construction of the proposed plant cell atlas [75]. Although advances in PTM analysis and *in planta* and *in vitro* interactomics have been leveraged, future work must incorporate the integration of spatial and temporal proteome dynamics. Rapid innovations can be leveraged via incorporation of technologies shown successful in mammalian systems, such as localization of organelle proteins by isotope tagging after differential ultracentrifugation (LOPIT-DC) for detailed analysis of spatially resolved proteomes [211]. By combining LOPIT-DC with an enrichable, isotopically labeled and MS-cleavable crosslinker (e.g. cyanurbiotindipropionylsuccinimide (CBDPS)), it may be possible to further extend the subcellular localization of LOPIT-DC to allow for quantitative analysis of protein conformations with cell-type and temporal specificity [206, 212]. Furthermore, as evidenced by the delineation of TOR signaling through the combined phosphoproteomic enrichment with AP-MS and XL-MS, the integration of multi-modal proteomic techniques is essential for comprehensive understanding of the plant proteome [210].

Another lagging line of evidence in plant proteomic analysis is the incorporation of imaging-MS (IMS) technologies; while IMS has been used for metabolomics in plants, it has only sparingly been employed for spatial protein analysis [213–217]. This is due in part to the inherent challenges for higher molecular weight compounds. The incorporation of high resolving power mass spectrometry (such as that achieved through 15T FTICR) had led to enhanced intact protein analysis for proteins [218]. Recent work has also benefited from the incorporation of ion mobility and/or on tissue enzymatic digestion to increase the sensitivity and dynamic range of spatial protein analysis, both of which could be applied in plant systems with minimal optimization [219–221]. While further increases in protein identification has occurred through pairing with LC-MS/MS, this approach requires bulk extraction to ensure sufficient sample size for analysis [222]. However, incorporating laser capture microdissection for sample preparation with automated nanodroplet processing, as has been

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applied with great success to mammalian tissues, could potentially be used for the relative quantitation of spatial proteomes across plant tissues [223]. Finally, the advancement of three dimensional quantitative IMS in mammalian systems also points toward a promising future in the spatial analysis of plant systems [224]. This has been successfully applied to the petals of *Bellis perennis* (daisies), where an autofocusing MALDI system enabled a lateral resolution of ≤10 μm [225]. However, more optimization is needed to integrate three dimensional IMS with protein detection to allow for spatial protein analysis.

Summary

- Plant proteins are the direct effectors of external stimuli, making proteomic analysis critical for understanding plant stress and homeostasis.
- Innovations in the quantitative analysis of phosphorylation and cysteine oxidation have revealed distinct signaling pathways across conserved protein networks, underscoring the need for further plant-focused investigations.
- Diverse interactomic techniques allow complementary data integration for both targeted and non-biased analysis of both protein interactions as well as quantitative complex dynamics.
- Future work must aim to further integrate proteomic techniques to enhance spatial and temporal resolution.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

Both authors contributed to the development of the manuscript, provided critical feedback, and reviewed and approved the final, submitted version of the manuscript.

Abbreviations

AMDSP, Azide-tag modified disuccinimidyl pimelate; AP-MS, affinity purification mass spectrometry; BN-PAGE, Blue-native polyacrylamide electrophoresis; CF-MS, cofractional mass spectrometry; CN-PAGE, clear-native polyacrylamide electrophoresis; Cys, cysteine; DTT, dithiothreitol; IAM, iodoacetamide; IMAC, immobilized metal affinity capture; IMS, imaging mass spectrometry; LOPIT-DC, localization of organelle proteins by isotope tagging after differential ultracentrifugation; MALDI, matrix-assisted laser desorption/ionization; MOAC, metal oxide affinity capture; NEM, *N*-ethylmaleimide; PL, proximity-dependent labeling; PolyMAC, polymer-metal affinity capture; PTMs, post-translational modifications; ROS, reactive oxygen species; SEC, size exclusion chromatography; TCEP, Tris(2-carboxyethyl)phosphine; XL-MS, crosslinking mass spectrometry.

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