Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Plant Biology

Advances in functional proteomics to study plant-pathogen interactions

James M. Elmore, Brianna D. Griffin and Justin W. Walley

Abstract

Pathogen infection triggers complex signaling networks in plant cells that ultimately result in either susceptibility or resistance. We have made substantial progress in dissecting many of these signaling events, and it is becoming clear that changes in proteome composition and protein activity are major drivers of plant-microbe interactions. Here, we highlight different approaches to analyze the functional proteomes of hosts and pathogens and discuss how they have been used to further our understanding of plant disease. Global proteome profiling can quantify the dynamics of proteins, post-translational modifications, and biological pathways that contribute to immune-related outcomes. In addition, emerging techniques such as enzyme activity–based profiling, proximity labeling, and kinase-substrate profiling are being used to dissect biochemical events that operate during infection. Finally, we discuss how these functional approaches can be integrated with other profiling data to gain a mechanistic, systems-level view of plant and pathogen signaling.

Addresses

Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA, 50014, USA

Corresponding authors: Walley, Justin W. (jwalley@iastate.edu); Elmore, James M. (jmelmore@iastate.edu)

Current Opinion in Plant Biology 2021, **63**:102061

This review comes from a themed issue on **Cell signaling and gene regulation**

Edited by **Hong Qiao** and **Anna N. Stepanova**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.pbi.2021.102061

Keywords

Immune signaling, Plant pathogen, Proteomics, Posttranslational modification.

Introduction

Proteins are the machines of the cell, driving nearly every aspect of organismal growth, reproduction, and stress responses. Although transcriptome analyses give a good indication of what the cell *can possibly do*, proteome analyses can yield a better picture of what the cell *is doing* at any given point in time. Mass spectrometry (MS)-based, quantitative proteomics is essential to understanding global changes in protein abundance and how the diversity of protein posttranslational modifications (PTMs) contribute to plant responses to pathogens. Over time, the field of proteomics has evolved from being primarily descriptive, generating lists of proteins or PTMs, to more functional and quantitative approaches that aim to understand the molecular mechanisms that regulate cellular activities [1]. Functional proteomics includes not only expression profiling under perturbation but also quantifying PTMs, characterizing the behavior of specific classes of proteins, determining activated enzyme states, and identifying and testing protein-protein interactions. These approaches can be integrated with other -omics data to generate multilevel network models of plant function. Thus, MS-based proteomics is a robust tool to not only generate hypotheses but also to test them in sophisticated ways not possible 10 years ago. In this review, we describe recent technological advances that enable functional proteomics and highlight their application toward studying plant-pathogen interactions.

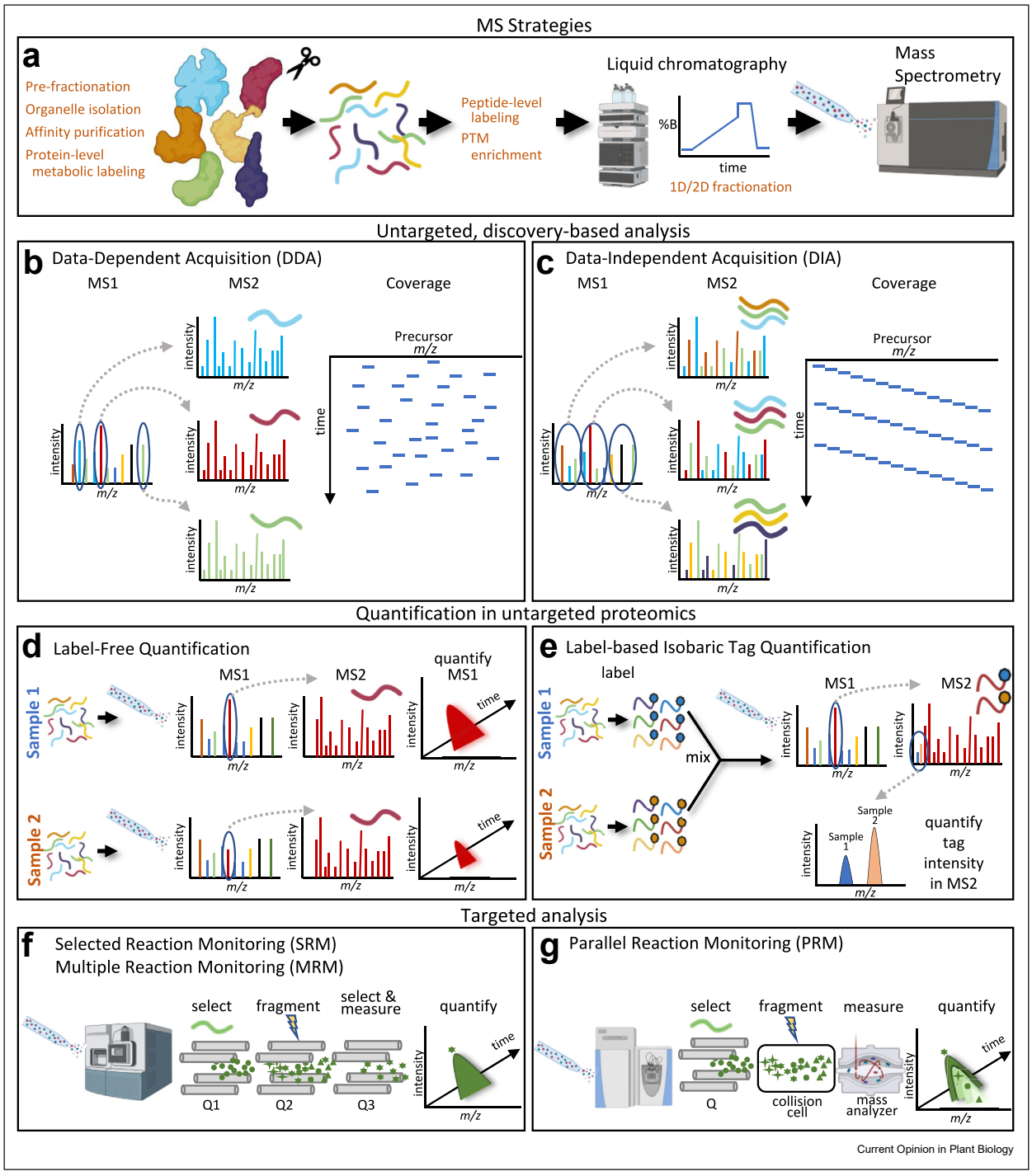
MS strategies

Many options exist for MS-based quantitative proteomics (Fig. 1a) [2]. Decisions on what strategies to use are influenced by the specific biological question(s), and the equipment/expertise available for sample analysis. Researchers interested in global-scale proteome changes during infection would likely choose an *untargeted analysis* (also termed "discovery" or "shotgun") approach to quantify as many proteins as possible. For researchers interested in accurate and precise quantification of the behavior of a specific protein, small group of proteins or specific PTM sites, a *targeted analysis* is preferred. In both approaches, researchers can decide on how the data are acquired and quantified, and these decisions will influence experimental design and sample processing.

Untargeted or discovery analysis: data-dependent and data-independent acquisition

Traditionally, data-dependent acquisition (DDA) has dominated untargeted proteomics (Fig. 1b). In DDA mode, MS1 scans identify the 10–25 most abundant peptide ions at each point of the chromatographic gradient. These precursor ions are sequentially selected

Fig. 1



Strategies in quantitative mass spectrometry-based proteomics. (a) A general sample processing and analysis workflow for a quantitative liquid chromatography mass spectrometry (LC-MS) experiment. Optional steps are indicated in orange text. (b) In data-dependent acquisition (DDA), the most abundant ions in MS1 scans during of the LC gradient are isolated individually and fragmented to generate MS2 spectra used for peptide identifications. The semistochastic nature of ion selection results in variable coverage of peptide species from run to run. (c) In data-independent acquisition (DIA), mass-to-charge (*m/z*) ranges are selected for fragmentation, yielding multiplexed MS2 spectra used for peptide identification and quantification. This approach results in consistent measurements and more uniform sample coverage. (d) Label-free quantification relies on comparing peptide amounts across different LC-MS runs. (e) Label-based quantification approaches, such as using isobaric mass tags, use chemical modifications to distinguish different samples. Modification allows the mixing of different samples and co-analysis in the same LC-MS run. (f) Targeted MS quantification using selected- or multiple-reaction monitoring (SRM/MRM) uses a triple quadrupole (Q-Q-Q) instrument to select specific precursor ions and measure specific fragment ions. (g) Targeted MS quantification using parallel reaction monitoring (PRM) uses a hybrid quadrupole-orbitrap or Q-time of flight instrument to select specific precursor ions, but all fragment ions are measured in the high-resolution mass analyzer.

and fragmented to acquire MS2 spectra. MS2 spectra in DDA are mostly pure in that each corresponds to single analyte (except in cases of chimeric spectra arising from co-isolation of ions with similar *m/z*). MS2 spectra are matched to a sequence database to identify specific peptides with various search engines using spectrum-centric scoring [3]. Recent advances in "open" search algorithms that use a wide precursor mass tolerance enhance our ability to identify PTMs and chemical modifications that can be missed by traditional narrow mass window searches [4]. A deep proteome survey of *Arabidopsis* developmental stages and immune responses to flg22, an immunogenic epitope of bacterial flagellin, used open searches to estimate that phosphorylation and acetylation each affect approximately 1.25% of the total identified protein content [5]. Ion selection in DDA is semistochastic and depends on the relative abundance and ionization efficiency of co-eluting peptides in the sample. Thus, usually the most abundant peptides are identified consistently from run to run. As a consequence, when analyzing a data set from multiple DDA runs, missing values are often encountered, which limits the statistical analysis of all identified proteins across a study.

Data-independent acquisition (DIA) approaches have emerged as a viable alternative to DDA (Fig. 1c). DIA-MS exhibits high quantitative accuracy and good coverage, even for low-abundance proteins, which alleviates the missing value problem [6]. Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) is one of the most popular DIA methods [7]. In SWATH-MS, all precursor ions in a narrow mass window (e.g. 400−425 *m/z*) are selected for fragmentation, yielding a highly multiplexed MS2 spectrum containing product ions from all co-eluting precursors in that mass window. The mass window is then shifted (i.e. 425−450 *m/z*, 450−475 *m/z*, etc.) in subsequent scans to cover the mass range of most tryptic peptides. This cycle is repeated over the course of the run to generate an unbiased map of detectable peptides in the sample. DIA data, as a result, are more consistent and reproducible than DDA [7]. Identifying peptides from complex DIA MS2 spectra can be challenging, and various peptide-centric and spectrum-centric approaches have been developed [6]. Some of these rely on using prior information from DDA data in the form of spectral libraries, but *in silico* generation of theoretical libraries and library-free approaches are accelerating the adoption of DIA-MS [6]. Another feature of DIA is the data can be queried retroactively, meaning that as spectral libraries and algorithms are developed, previous DIA results can be reanalyzed to increase protein quantification.

Recent studies have used DIA-MS to study plant-pathogen interactions. SWATH-MS analysis of apoplastic fluid from susceptible barley leaves infected with *Pyrenophora teres* f. *teres* quantified over 1000 proteins and found strong upregulation of multiple classes of pathogenesis-related (PR) proteins, including antifungal thaumatin-like PR-5 proteins [8]. This finding suggests that the fungus deploys mechanisms to overcome the activities of these defense proteins. A similar strategy identified over 2000 proteins from tomato leaves at 4-, 8-, and 24-h after infection with *Pseudomonas syringae* pv. *tomato* DC3000. Most of the detected changes occurred at 8 and 24 h, including a strong upregulation in proteins associated with energy generation, immune response, and redox processes and a downregulation of carbon fixation in the chloroplast [9]. SWATH-MS also uncovered a downregulation of photosynthesis and an increase in flavonoid and jasmonic acid biosynthesis associated with rice resistance to leaffolder insect infestation [10]. As new mass spectrometers with enhanced resolution and scan speeds are introduced, DIA-MS has the potential to overtake DDA-MS as the method of choice for discovery-based proteomics.

Quantification and multiplexing in untargeted proteomics: label-free and label-based approaches

Many researchers are interested in quantifying the molecular changes that drive phenotypes such as disease resistance or susceptibility. MS-based protein or peptide quantification can be divided into label-free and label-based approaches [11]. Label-free methods for DDA data include MS1-based quantification of extracted ion chromatograms (XICs) from precursor peptides (Fig. 1d), MS2 count-based methods such as spectrum counting, and combinations of both [12]. Peptides identified in label-free DIA data are quantified using MS2 and/or MS1 XICs [6]. One key consideration for label-free strategies is the inability to multiplex samples can lead to run-to-run variability, which can lower quantitative precision.

Label-based quantification strategies are designed around the ability to multiplex, meaning that different samples are chemically modified, combined, and co-analyzed in the same liquid chromatography mass spectrometry (LC-MS) run. This feature can eliminate important sources of variability, greatly reduce missing data, and substantially reduce demands on instrument time. Stable isotope labeling by amino acids in cell culture (SILAC) has been performed in plants, but their autotrophic nature leads to incomplete incorporation of heavy isotopes under most growth conditions [13]. An alternative to SILAC to metabolically label proteins is to use ^{15}N labeling [13]. Most often, plant samples are labeled at the peptide level using chemical labeling via reductive dimethylation [14], isotope-coded affinity tags [15], or isobaric mass tags, such as iTRAQ [16] or TMT [17] (Fig. 1e). These strategies vary on the degree of multiplexing that can be achieved and whether peptides are quantified at the MS1 or MS2 level. Furthermore, the number of co-analyzed samples can be extended by combining different types of labels [18]. In addition to reducing variability, sample multiplexing can substantially reduce instrument time, especially in

studies with hundreds of samples to be analyzed. In general, MS2 level isobaric tag-based quantification is highly precise but less accurate, especially for large fold changes due to ratio compression, whereas label-free XIC-based quantification yields less precise but more accurate measurements [19,20].

Targeted quantification by reaction monitoring

MS-based targeted quantification strategies program the MS to isolate and quantify a small group (usually less than 100) of peptides. Selected- and multiple-reaction monitoring (SRM/MRM) use a triple quadrupole (Q-Q-Q) instrument to monitor and isolate specific, preselected precursor ions in Q1 (Fig. 1f). Precursors are then fragmented in Q2, and specific product ion XICs are measured in Q3. This dual-stage filtering empowers excellent sensitivity for quantifying even very low-abundance peptides [21]. SRM/MRM assay development can be resource intensive and requires the selection of proteotypic peptides, optimal transitions (precursor ion pairs>fragment ion pairs), and validation before quantitative experiments. Similar to SRM/MRM, parallel reaction monitoring (PRM) uses the quadrupole of Q-Orbitrap or Q-TOF instruments as a mass filter for targeted precursors, but all generated fragment ions are monitored in the high-resolution mass analyzer (Fig. 1g) [22]. This obviates the need to pick specific transitions up-front. In all cases, the use of isotopically labeled synthetic peptides can assist method development, validation, and quantification [23].

The power of MS-based targeted approaches lies in the ability to quantify nearly any protein or PTM site using short chromatographic gradients. Recent targeted approaches have characterized phosphorylation mechanisms that regulate the NAPDH oxidase RBOHD activity during pattern- and effector-triggered immune (PTI/ETI) responses [24–26]. SRM identified antagonism between phospho- and acetyl-sites that regulate activity of the immune receptor RRS1-R in *Arabidopsis* [27]. SRM confirmed *in planta* BIK1-dependent phosphorylation of the calcium channel OSCA1.3 at serine 54, which is required for stomatal closure during PTI [28]. Thus, targeted MS strategies can replace Western blotting with custom antibodies and can speed confirmation of *in vivo* protein and PTM dynamics [29].

Functional proteomics approaches

Purify to probe proteins participating in particular processes

Reducing sample complexity is useful for studying specific aspects of plant-microbe interactions because it can boost identifications of low-abundance plant and/or pathogen proteins. This can be achieved by purifying specific proteins, organelles, or structures during sample preparation and/or two-dimensional peptide chromatography during LC-MS. Affinity purification/affinity enrichment (AP/AE) using antibodies or epitopes to purify target protein(s) of interest is useful to not only quantify low-abundance proteins but also to identify new PTM sites and interacting protein partners [30,31]. AE-MS using an anti-all-WRKY antibody quantified the dynamics of the WRKY transcription factor family upon flg22 elicitation [32]. DeBlasio et al. used a similar strategy to identify host proteins that associate with *Potato leafroll virus* and used virus mutants to characterize subnetworks of protein-protein interactions that control different stages of the viral infection cycle [33–36].

Recent work has identified tissue-type and organelle-specific dynamics during disease progression. Quantitative analysis of purified tomato leaf nuclei revealed hundreds of proteins whose abundance dynamically changes during *Phytophthora* infection and led to the identification of AT-Hook-Like DNA-binding proteins as positive regulators of the immune response to *Phytophthora capsici* [37]. Analysis of the barley leaf epidermal proteome has characterized proteins that modulate plant susceptibility to powdery mildew, which infects only epidermal cells [38]. Fungal secretome analyses have identified putative virulence factors [39], and proteomics of apoplastic fluid has characterized both pathogen and plant proteins that are secreted during infection [40]. Recently, extracellular vesicles from plants and pathogens have emerged as key mediators of infection. Studies have revealed the identity of the protein cargo contained within these secreted vesicles, including important immune-related proteins from the plant side and virulence-associated proteins from the pathogen [41–43]. These various pre-MS enrichment strategies are critical to identify not only specific plant organellar proteins for more focused studies but can also enable the quantification of pathogen proteins that can be present at low levels in whole-plant samples.

Proximity labeling

Proximity labeling is a relatively new technology for determining protein-protein interactions and offers a powerful approach to determine biochemical events occurring during plant-microbe interactions. Proximity labeling is complimentary to AP-MS strategies [30]. AP-MS can capture high-affinity interactions but not weak transient interactions and has low efficiency for recovering membrane proteins [44]. Proximity labeling overcomes these inherent limitations of AP-MS. Proteins of interest are fused with a catalytic enzyme that, in the presence of substrate, rapidly covalently tag proximal proteins. The most commonly used enzymes are biotin ligase variants (BioID, BioID2, TurboID, and mini-TurboID) and ascorbate peroxidase (APEX), which both result in biotinylation of proximal proteins [45–47]. However, because APEX requires the addition of toxic H_2O_2 and biotin-phenol, biotin ligase variants are best suited for *in vivo* studies. Finally, because of fast kinetics, proximity labeling enables identification of weak and

transient interactors and is able to effectually recover interactors of membrane proteins [48–52].

As proximity labeling is an emerging technique, it is just beginning to shed light on interactors of proteins involved in plant immune signaling. Conlan et al. used BioID to identify proteins that interact with AvrPto, an effector protein in *P. syringae*, to further elucidate plant immune responses [53]. Das et al. used BioID to identify transient interacting proteins to a tobacco mosaic virus replicase [54]. Zhang et al. identified the interacting proteins to N, a resistance protein that recognizes tobacco mosaic virus. They found the biotin ligase variant TurboID to outperform BioID and BioID2 and demonstrated the utility of TurboID for identifying transient interactors of the N immune receptor. Among the N-interacting proteins, the E3 ubiquitin ligase UBR7 was shown to negatively regulate N protein levels and *N*-mediated resistance to TMV [55]. Proximity labeling will surely complement other protein-protein interaction screens to generate more complete host and host–microbe interactomes.

PTM profiling

Protein PTMs are covalent attachments of various functional groups to specific amino acid residues. PTMs can modulate enzyme activity, protein structure, and interaction with other proteins, thus impacting protein function and/or localization within the cell. MS-based proteomics is currently the method of choice to identify and quantify PTMs at the proteome and individual protein levels. The vast array of different PTMs and potentially modified sites suggests a level of complexity to protein regulation of which we are only scratching the surface [56–58].

Phosphorylation has been the most intensively studied PTM owing to its prevalent occurrence, relative ease in purification of phosphopeptides, and the extensive involvement of kinase signaling networks in plant immunity [59]. Refinements to phospho-enrichment strategies, peptide separations, and MS technology improvement have enabled the quantification of 10,000 to >30,000 phosphosites in a single study. Typically, one- or multi-stage enrichment strategies using immobilized metal-affinity or metal oxide–affinity chromatography are used to achieve comprehensive coverage of phosphorylation events [60,61]. Recent large-scale phosphoproteome analyses have uncovered dynamic phosphorylation events during PTI and ETI that regulate plant immunity to pathogens [25,62]. Phosphoproteomics combined with mutant analyses can reveal direct and indirect targets of phosphosignaling cascades [63]. Comparison of the cytoplasmic and chromatin-associated phosphoproteomes of mitogen-activated protein kinase (MAPK) knockout lines identified shared and specific target proteins, including new kinases and chromatin-associated proteins that regulate in immune signaling [64,65].

Large-scale profiling of other PTMs during plant-microbe interactions is becoming more common. Enrichment of acetylated and ubiquitinated peptides is typically performed using antibody affinity-based enrichment [66–69]. Recent studies have identified hundreds of ubiquitin-modified proteins during PTI responses in rice [70] and *Arabidopsis* [71]. In an alternate purification strategy, Ma et al. used a genetically encoded tagged ubiquitin protein and two-step purification to isolate ubiquitin-conjugated proteins upon treatment with flg22 [72]. They found many known immune proteins to be ubiquitinylated during flg22 responses and identified receptor-like kinase RKL1, ubiquitin-conjugating enzyme UBC13, and proteasome component RPN8b as negative regulators of PTI responses [72]. Quantitative acetylome profiling of maize in response to HC-toxin produced by *Cochliobolus carbonum* revealed hyperacetylation of many proteins involved in transcriptional regulation, including transcription factors, co-repressors, and chromatin remodeling enzymes [67]. In addition, several recent studies have identified thousands of acetylated proteins in diverse plant pathogens, although the impact of these PTMs on pathogenicity remains to be studied [73–75]. Analysis of reversible cysteine oxidation in *Arabidopsis* uncovered distinct, time-dependent patterns of protein oxidation during ETI [76]. These and other studies lay the foundation for understanding the functional roles of specific PTMs and PTM cross-talk during plant-microbe interactions.

Activity-based protein profiling

Activity-based protein profiling (ABPP) is a powerful technique to characterize enzyme active states. ABPP uses small-molecule tags that covalently bond to enzyme active sites [77]. The tags are linked to various reporter groups, such as fluorophores for gel-based visualization, biotin for affinity-based purification, or reactive azide or alkyne moieties for click-based reporter additions. ABPP tags have been developed for a wide variety of enzymes, including distinct subclasses of cysteine proteases, proteasome subunits, serine hydrolases, vacuolar processing enzymes, glycosyl hydrolases, and ATP-binding proteins [77].

Because ABPP probes label enzymes in their active states, they are an excellent tool to survey functional subproteomes of plants and pathogens. Franco et al. identified global reprogramming of peroxidase and serine protease activity in the phloem of citrus trees infected with the citrus greening pathogen [78]. Combined ABPP and abundance profiling of apoplastic fluid from tomato revealed activation of the cysteine proteases Rcr3 (Required for *Cladosporium fulvum* resistance 3) and Pip1 (*Phytophthora* inhibited protease 1), post-translational activation of specific P69 subtilase serine

proteases, and a general inhibition of GDSL-lipases during *Ralstonia solanacearum* infection [79]. Rcr3 and Pip1 are related proteases that have differential roles in immunity to fungal, oomycete, and bacterial pathogens [80]. ABPP demonstrated that Rcr3 is processed *in planta* by P69B and other host subtilases to convert it to a catalytically active form [81]. ABPP of plant enzymes can also identify pathogen virulence factors that inhibit host immune-related enzymes [82]. Glycosidase activity profiling revealed a strong decrease in *Nicotiana benthamiana* β-galactosidase BGAL1 activity during *P. syringae* infection and revealed the presence of small inhibitor molecule produced by *Pseudomonas* during infection [83]. Further investigation discovered a mechanism by which BGAL1 contributes to plant immunity by hydrolyzing glycans decorating bacterial flagellin, thus exposing it to host proteases that process it to immunogenic peptides that are recognized by FLS2 RLKs in *Nicotiana* and *Arabidopsis* [83].

N-terminomics for protease substrate identification

In addition to profiling active proteases, identification of protease targets is of key interest because proteolysis acts at many levels of infection, including host immune suppression, pathogen recognition, peptide hormone signaling, priming, and the hypersensitive response [84—86]. Recent efforts have exploited the ability of diverse plant species to recognize the *P. syringae* effector protease AvrPphB to engineer new disease resistance specificities to unrelated pathogen proteases [87—89]. To apply this approach to new pathosystems, the target cleavage sequence of pathogen proteases must be identified.

Some of the most widely used methods to identify protease substrates include TAILs (terminal amine isotopic labeling of substrates) [90], COFRADIC (combined fractional diagonal chromatography) [91], and ChaFRADIC (charge-based fractional diagonal chromatography) [92]. All three methods rely on a first step to block or label primary amines (exposed N-termini and lysines) at the protein level, followed by enzymatic digestion, which yields new primary amines that are subsequently depleted from the sample. This negative selection step enriches native protein N-termini and N-terminal proteolytic products, which are analyzed via LC-MS. These techniques can link host proteases with different immune pathways, as well as identify effector protease substrates, ultimately unraveling mechanisms of disease resistance and susceptibility.

Kinase substrate identification and kinase signaling networks

Kinase-signaling networks serve essential functions during plant-microbe interactions. Protein microarrays are one approach to uncover kinase substrates. Using protein microarrays, Popescu et al. were able to reconstruct a signaling network comprised of 10 MAPKs and 570 putative substrates [93]. Another approach to directly identify kinase substrates is multiplexed assay for kinase specificity (MAKS) [94]. For MAKS, protein extracts are incubated with a recombinantly produced kinase. After incubation, the samples are multiplexed using isobaric tags and analyzed by MS using standard phosphoproteomic workflows. Thus, MAKS allows for high-throughput proteome-wide identification of direct kinase substrates, including substrates of the receptor-like kinases LYK3 and NORK, which are required for nodulation and arbuscular mycorrhizal associations in *Medicago truncatula* [94]. A conceptually similar method is kinase assay linked phosphoproteomics (KALIP 2.0), where phosphopeptides are extracted from plant tissue, dephosphorylated, and then incubated *in vitro* with recombinant kinases [95]. This method resulted in the identification of 5,075 putative targets of nine kinases, including MPK6 and CPK11, after incubation with the elicitor flg22 [95].

Computational reconstruction of kinase-signaling networks from phosphoproteomics data sets offers another approach to predict the relationship of kinases and potential substrates. Activation sites of protein kinases are regulated by phosphorylation in their activation loop (A-loop) [96,97]. Because A-loop phosphorylation is necessary for kinase activation, phosphosite intensity in the A-loop can be used to infer kinase activity. Using this information, various approaches have reconstructed kinase signaling networks by correlating phosphosite level with kinase activation state [98—100].

Looking ahead: integration of orthogonal data sets for systems-level predictions

Host and pathogen signaling during infection is coordinated at multiple molecular, temporal, and spatial scales. One key challenge moving forward is to integrate different types of functional proteomics data with orthogonal genome, interactome, transcriptome, metabolome, and phenome information to generate predictive models of the mechanisms underlying disease resistance and susceptibility [101,102]. One issue is data from different sources can have disparate properties. Current proteomics data lack the depth of most transcriptome studies, and the "bottom-up" approach of analyzing peptides can result in uncertainty in protein assignments. Furthermore, the diversity of functional proteoforms generated from the same protein-coding gene means it is hard to derive a single, protein-level measurement that can be matched to a gene or transcript. In addition, different -omics data can relate to each other in different ways, so there is no "one size fits all" solution for data integration. There are a multitude of available approaches, ranging from correlation/cluster analyses and pathway mapping to machine learning and mathematical modeling [101—103]. It has been shown that

the predictive power of gene regulatory networks can be improved by combining transcription factor protein and phosphosite abundance to predict target transcript regulation [104]. Thus, true integrative analysis differs from parallel data collection and interpretation and exploits the synergistic properties of different data types to discover novel biological relationships [105].

Integrated transcriptome, protein interactome, and mutant analyses uncovered *Arabidopsis* protein subnetworks that contribute to quantitative disease resistance to *Xanthomonas campestris* [106]. Ding et al. integrated transcriptomic data and Genome-Wide Association Study (GWAS) with enzyme assays, proteomics, and mutant analysis to elucidate the kauralexin biosynthesis pathway that contributes to *Fusarium* stalk rot resistance in maize [107]. Time-resolved, multiomics analyses revealed coordinated molecular changes in *Candidatus liberibacter asiaticus*—infected citrus trees before the development of citrus greening symptoms and identified biomarkers for early detection [108,109]. Sekiya et al. combined proteome and metabolome measurements with weighted gene co-expression network analysis in rust-resistant and rust-susceptible eucalyptus to predict the protein modules that regulate metabolites associated with immunity and disease [110]. Nobori et al. integrated transcriptomic and proteomic data of *P. syringae* isolated from wild-type and mutant *Arabidopsis* to discover bacterial virulence mechanisms that are suppressed by host salicylic acid signaling [111]. These and other studies exemplify the power of multiomics data integration to generate a systems-level view of plant-microbe interactions.

Conclusion

The functional proteomics approaches described above are just some of the technologies that are being applied to gain new insights into diverse plant pathosystems. Global proteome profiling during infection can identify specific proteins, PTMs, and biological pathways that contribute to disease resistance and susceptibility. Activity-based profiling, proximity labeling, and kinase-substrate profiling characterize specific immune signaling subnetworks and pathogenic strategies that operate during infection. One challenging, but exciting, prospect is integrating these data with other -omic data to associate molecular traits with each other and connect mechanisms with immune outcomes. Together, these systems approaches can guide breeding and engineering efforts, leading to the development of disease-resistant crops and improved global food security.

Funding

This work was supported by the National Science Foundation (IOS-1759023 and MCB-1818160) and National Institutes of Health (R01GM120316).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Monti C, Zilocchi M, Colugnat I, Alberio T: **Proteomics turns functional**. *J Proteom* 2019, **198**:36–44.

2. Schubert OT, Röst HL, Collins BC, Rosenberger G, Aebersold R: **Quantitative proteomics: challenges and opportunities in basic and applied research**. *Nat Protoc* 2017, **12**:1289–1294.

3. Verheggen K, Ræder H, Berven FS, Martens L, Barsnes H, Vaudel M: **Anatomy and evolution of database search engines—a central component of mass spectrometry based proteomic workflows**. *Mass Spectrom Rev* 2020, **39**:292–306.

4. * Yu F, Teo GC, Kong AT, Haynes SE, Avtonomov DM, Geiszler DJ, Nesvizhskii AI: **Identification of modified peptides using localization-aware open search**. *Nat Commun* 2020, **11**:4065.
The authors report advancements to the open-search engine MSFragger that allow the identification of more of the "dark matter" of discovery proteomics—the multitude of MS2 spectra that are not matched to peptide sequences using traditional search algorithms. Open searches can significantly enhance the identification of modified peptides in DDA data.

5. ** Bassal M, Abukhalaf M, Majovsky P, Thieme D, Herr T, Ayash M, Tabassum N, Al Shweiki MR, Proksch C, Hmedat A, *et al.*: **Reshaping of the Arabidopsis thaliana proteome landscape and co-regulation of proteins in development and immunity**. *Mol Plant* 2020, **13**:1709–1732.
This study performed label-free proteome profiling of different *Arabidopsis* tissues across developmental stages and in response to flg22, identifying more than 16,000 proteins in total. This is one of the deepest proteomics analyses of PTI responses in plants, and the authors estimated nearly one-third of the cellular proteome is remodeled after 16 h of flg22 exposure.

6. Zhang F, Ge W, Ruan G, Cai X, Guo T: **Data-independent acquisition mass spectrometry-based proteomics and software tools: a glimpse in 2020**. *Proteomics* 2020, **20**:1900276.

7. Ludwig C, Gillet L, Rosenberger G, Amon S, Collins BC, Aebersold R: **Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial**. *Mol Syst Biol* 2018, **14**:e8126.

8. Hassett K, Ellwood SR, Zulak KG, Muria-Gonzalez MJ: **Analysis of apoplastic proteins expressed during net form net blotch of barley**. *J Plant Dis Prot* 2020, **127**:683–694.

9. * Fan K-T, Wang K-H, Chang W-H, Yang J-C, Yeh C-F, Cheng K-T, Hung S-C, Chen Y-R: **Application of data-independent acquisition approach to study the proteome change from early to later phases of tomato pathogenesis responses**. *Int J Mol Sci* 2019, **20**.
This study used SWATH-MS to characterize early and late proteome dynamics of tomato in response to virulent *P. syringae*, with around 90% of proteins identified across all timepoints.

10. Cheah BH, Lin H-H, Chien H-J, Liao C-T, Liu L-YD, Lai C-C, Lin Y-F, Chuang W-P: **SWATH-MS-based quantitative proteomics reveals a uniquely intricate defense response in Cnaphalocrocis medinalis-resistant rice**. *Sci Rep* 2020, **10**:6597.

11. Anand S, Samuel M, Ang C-S, Keerthikumar S, Mathivanan S, Label-Based, Strategies Label-Free: **For protein quantitation**. In *Proteome bioinformatics*. Edited by Keerthikumar S, Mathivanan S, New York: Springer; 2017:31–43.

12. Blein-Nicolas M, Zivy M: **Thousand and one ways to quantify and compare protein abundances in label-free bottom-up proteomics**. *Biochim Biophys Acta Protein Proteonomics* 2016, **1864**:883–895.

13. Matthes A, Köhl K, Schulze WX: **SILAC and alternatives in studying cellular proteomes of plants**. In *Stable isotope labeling by amino acids in cell culture (SILAC): methods and protocols*. Edited by Warscheid B, New York: Springer; 2014:65–83.

14. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJR: **Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics**. *Nat Protoc* 2009, **4**:484–494.

15. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R: **Quantitative analysis of complex protein mixtures using isotope-coded affinity tags**. *Nat Biotechnol* 1999, **17**:994–999.

16. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, *et al.*: **Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents**. *Mol Cell Proteomics* 2004, **3**:1154–1169.

17. Li J, Van Vranken JG, Pontano Vaites L, Schweppe DK, Huttlin EL, Etienne C, Nandhikonda P, Viner R, Robitaille AM, Thompson AH, *et al.*: **TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples**. *Nat Methods* 2020, **17**:399–404.

18. Aggarwal S, Talukdar NC, Yadav AK: **Advances in higher order multiplexing techniques in proteomics**. *J Proteome Res* 2019, **18**:2360–2369.

19. Savitski MM, Mathieson T, Zinn N, Sweetman G, Doce C, Becher I, Pachl F, Kuster B, Bantscheff M: **Measuring and managing ratio compression for accurate iTRAQ/TMT quantification**. *J Proteome Res* 2013, **12**:3586–3598.

20. * Hogrebe A, von Stechow L, Bekker-Jensen DB, Weinert BT, Kelstrup CD, Olsen JV: **Benchmarking common quantification strategies for large-scale phosphoproteomics**. *Nat Commun* 2018, **9**:1045.
This study performed comprehensive benchmarking of DDA quantification strategies including label-free as well as MS2 and MS3 measured TMT. They found that despite ratio compression, MS2-based TMT enabled detection of the most differentially regulated phosphopeptides.

21. Picotti P, Aebersold R: **Selected reaction monitoring–based proteomics: workflows, potential, pitfalls and future directions**. *Nat Methods* 2012, **9**:555–566.

22. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ: **Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics**. *Mol Cell Proteomics* 2012, **11**:1475–1488.

23. Picotti P, Rinner O, Stallmach R, Dautel F, Farrah T, Domon B, Wenschuh H, Aebersold R: **High-throughput generation of selected reaction-monitoring assays for proteins and proteomes**. *Nat Methods* 2010, **7**:43–46.

24. Kadota Y, Sklenar J, Derbyshire P, Stransfeld L, Asai S, Ntoukakis V, Jones JD, Shirasu K, Menke F, Jones A, *et al.*: **Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity**. *Mol Cell* 2014, **54**:43–55.

25. Kadota Y, Liebrand TWH, Goto Y, Sklenar J, Derbyshire P, Menke FLH, Torres M-A, Molina A, Zipfel C, Coaker G, *et al.*: **Quantitative phosphoproteomic analysis reveals common regulatory mechanisms between effector- and PAMP-triggered immunity in plants**. *New Phytol* 2019, **221**:2160–2175.

26. Kimura S, Hunter K, Vaahtera L, Tran HC, Citterico M, Vaattovaara A, Rokka A, Stolze SC, Harzen A, Meißner L, *et al.*: **CRK2 and C-terminal phosphorylation of NADPH oxidase RBOHD regulate reactive oxygen species production in Arabidopsis**. *Plant Cell* 2020, **32**:1063.

27. Guo H, Ahn H-K, Sklenar J, Huang J, Ma Y, Ding P, Menke FLH, Jones JDG: **Phosphorylation-regulated activation of the Arabidopsis RRS1-R/RPS4 immune receptor complex reveals two distinct effector recognition mechanisms**. *Cell Host Microbe* 2020, **27**:769–781.e6.

28. Thor K, Jiang S, Michard E, George J, Scherzer S, Huang S, Dindas J, Derbyshire P, Leitão N, DeFalco TA, *et al.*: **The calcium-permeable channel OSCA1.3 regulates plant stomatal immunity**. *Nature* 2020, **585**:569–573.

29. Aebersold R, Burlingame AL, Bradshaw RA: **Western blots versus selected reaction monitoring assays: time to turn the tables?** *Mol Cell Proteomics* 2013, **12**:2381–2382.

30. Morris JH, Knudsen GM, Verschueren E, Johnson JR, Cimermancic P, Greninger AL, Pico AR: **Affinity purification–mass spectrometry and network analysis to understand protein-protein interactions**. *Nat Protoc* 2014, **9**:2539–2554.

31. Keilhauer EC, Hein MY, Mann M: **Accurate protein complex retrieval by affinity enrichment mass spectrometry (AE-MS) rather than affinity purification mass spectrometry (AP-MS)**. *Mol Cell Proteomics* 2015, **14**:120–135.

32. * Birkenbihl RP, Kracher B, Ross A, Kramer K, Finkemeier I, Somssich IE: **Principles and characteristics of the Arabidopsis WRKY regulatory network during early MAMP-triggered immunity**. *Plant J* 2018, **96**:487–502.
The authors used affinity enrichment of WRKY transcription factors using and anti-all-WRKY antibody to measure protein levels during flg22 treatment. Combined with RNA-seq and ChIP-seq data, the results support a model where, in an uninduced state, constitutively expressed WRKYs act as repressors of flg22-induced WRKY gene promoters and are rapidly displaced on flg22 elicitation.

33. DeBlasio SL, Johnson R, Mahoney J, Karasev A, Gray SM, MacCoss MJ, Cilia M: **Insights into the polerovirus–plant interactome revealed by coimmunoprecipitation and mass spectrometry**. *MPMI (Mol Plant-Microbe Interact)* 2014, **28**:467–481.

34. DeBlasio SL, Johnson R, Sweeney MM, Karasev A, Gray SM, MacCoss MJ, Cilia M: **Potato leafroll virus structural proteins manipulate overlapping, yet distinct protein interaction networks during infection**. *Proteomics* 2015, **15**:2098–2112.

35. DeBlasio SL, Johnson RS, MacCoss MJ, Gray SM, Cilia M: **Model system-guided protein interaction mapping for virus isolated from phloem tissue**. *J Proteome Res* 2016, **15**:4601–4611.

36. DeBlasio SL, Xu Y, Johnson RS, Rebelo AR, MacCoss MJ, Gray SM, Heck M: **The interaction dynamics of two potato leafroll virus movement proteins affects their localization to the outer membranes of mitochondria and plastids**. *Viruses* 2018, **10**:585.

37. Howden AJM, Stam R, Martinez Heredia V, Motion GB, Ten Have S, Hodge K, Marques Monteiro Amaro TM, Huitema E: **Quantitative analysis of the tomato nuclear proteome during Phytophthora capsici infection unveils regulators of immunity**. *New Phytol* 2017, **215**:309–322.

38. Lambertucci S, Orman KM, Das Gupta S, Fisher JP, Gazal S, Williamson RJ, Cramer R, Bindschedler LV: **Analysis of barley leaf epidermis and extrahaustorial proteomes during powdery mildew infection reveals that the PR5 thaumatin-like protein TLP5 is required for susceptibility towards Blumeria graminis f. sp. hordei**. *Front Plant Sci* 2019, **10**:1138.

39. Vincent D, Rafiqi M, Job D: **The multiple facets of plant–fungal interactions revealed through plant and fungal secretomics**. *Front Plant Sci* 2020, **10**:1626.

40. Gupta R, Lee SE, Agrawal GK, Rakwal R, Park S, Wang Y, Kim ST: **Understanding the plant-pathogen interactions in the context of proteomics-generated apoplastic proteins inventory**. *Front Plant Sci* 2015, **6**:352.

41. Rutter BD, Innes RW: **Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins**. *Plant Physiol* 2017, **173**:728.

42. Bleackley MR, Samuel M, Garcia-Ceron D, McKenna JA, Lowe RGT, Pathan M, Zhao K, Ang C-S, Mathivanan S,

Anderson MA: **Extracellular vesicles from the cotton pathogen Fusarium oxysporum f. sp. vasinfectum induce a phytotoxic response in plants**. *Front Plant Sci* 2020, **10**:1610.

43. Hill EH, Solomon PS: **Extracellular vesicles from the apoplastic fungal wheat pathogen Zymoseptoria tritici**. *Fungal Biol. Biotechnol.* 2020, **7**:13.

44. Qin W, Cho KF, Cavanagh PE, Ting AY: **Deciphering molecular interactions by proximity labeling**. *Nat Methods* 2021, **18**: 133–143.

45. Roux KJ, Kim DI, Raida M, Burke B: **A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells**. *JCB (J Cell Biol)* 2012, **196**: 801–810.

46. Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY: **Directed evolution of APEX2 for electron microscopy and proximity labeling**. *Nat Methods* 2015, **12**: 51–54.

47. Branon TC, Bosch JA, Sanchez AD, Udeshi ND, Svinkina T, Carr SA, Feldman JL, Perrimon N, Ting AY: **Efficient proximity labeling in living cells and organisms with TurboID**. *Nat Biotechnol* 2018, **36**:880–887.

48. Khan M, Youn J-Y, Gingras A-C, Subramaniam R, Desveaux D: **In planta proximity dependent biotin identification (BioID)**. *Sci Rep* 2018, **8**:9212.

49. Mair A, Xu S-L, Branon TC, Ting AY, Bergmann DC: **Proximity labeling of protein complexes and cell-type-specific organellar proteomes in Arabidopsis enabled by TurboID**. *eLife* 2019, **8**:e47864.

50. Arora D, Abel NB, Liu C, Damme PV, Yperman K, Eeckhout D, Vu LD, Wang J, Tornkvist A, Impens F, *et al.*: **Establishment of proximity-dependent biotinylation approaches in different plant model systems**. *Plant Cell* 2020, **32**:3388–3407.

51. Huang A, Tang Y, Shi X, Jia M, Zhu J, Yan X, Chen H, Gu Y: **Proximity labeling proteomics reveals critical regulators for inner nuclear membrane protein degradation in plants**. *Nat Commun* 2020, **11**:3284.

52. Yang X, Wen Z, Zhang D, Li Z, Li D, Nagalakshmi U, Dinesh-Kumar SP, Zhang Y: **Proximity labeling: an emerging tool for probing in planta molecular interactions**. *Plant Commun.* 2020, https://doi.org/10.1016/j.xplc.2020.100137.

53. Conlan B, Stoll T, Gorman JJ, Saur I, Rathjen JP: **Development of a rapid in planta BioID system as a probe for plasma membrane-associated immunity proteins**. *Front Plant Sci* 2018, **9**.

54. Das PP, Macharia MW, Lin Q, Wong S-M: **In planta proximity-dependent biotin identification (BioID) identifies a TMV replication co-chaperone NbSGT1 in the vicinity of 126 kDa replicase**. *J Proteom* 2019, **204**:103402.

55. * Zhang Y, Song G, Lal NK, Nagalakshmi U, Li Y, Zheng W, Huang P, Branon TC, Ting AY, Walley JW, *et al.*: **TurboID-based proximity labeling reveals that UBR7 is a regulator of N NLR immune receptor-mediated immunity**. *Nat Commun* 2019, **10**: 3252.
This was the first published report of TurboID in plant systems. It is also shown that UBR7 directly interacts with and mediates the turnover N during N-mediated resistance to TMV.

56. He Z, Huang T, Ao K, Yan X, Huang Y: **Sumoylation, Phosphorylation, and Acetylation Fine-Tune the turnover of plant immunity components mediated by ubiquitination**. *Front Plant Sci* 2017, **8**:1682.

57. Csizmok V, Forman-Kay JD: **Complex regulatory mechanisms mediated by the interplay of multiple post-translational modifications**. *Curr Opin Struct Biol* 2018, **48**:58–67.

58. Zhang Y, Zeng L: **Crosstalk between ubiquitination and other post-translational protein modifications in plant immunity**. *Plant Commun.* 2020, **1**:100041.

59. * Willems P, Horne A, Van Parys T, Goormachtig S, De Smet I, Botzki A, Van Breusegem F, Gevaert K: **The Plant PTM Viewer, a central resource for exploring plant protein modifications**. *Plant J* 2019, **99**:752–762.
This newly developed, searchable repository for plant PTMs holds experimental data for 19 PTMs across five model plant species.

60. Zhang T, Schneider JD, Zhu N, Chen S: **Identification of MAPK substrates using quantitative phosphoproteomics**. In *Plant pattern recognition receptors: methods and protocols*. Edited by Shan L, He P, New York: Springer; 2017:133–142.

61. Song G, Montes C, Walley JW: **Quantitative profiling of protein abundance and phosphorylation state in plant tissues using tandem mass tags**. In *Plant proteomics: methods and protocols*. Edited by Jorrin-Novo JV, Valledor L, Castillejo MA, Rey M-D, US: Springer; 2020:147–156.

62. Kohorn BD, Hoon D, Minkoff BB, Sussman MR, Kohorn SL: **Rapid oligo-galacturonide induced changes in protein phosphorylation in Arabidopsis**. *Mol Cell Proteomics* 2016, **15**: 1351–1359.

63. Song G, Brachova L, Nikolau BJ, Jones AM, Walley JW: **Heterotrimeric G-protein-dependent proteome and phosphoproteome in unstimulated Arabidopsis roots**. *Proteomics* 2018, **18**:1800323.

64. Rayapuram N, Bigeard J, Alhoraibi H, Bonhomme L, Hesse A-M, Vinh J, Hirt H, Pflieger D: **Quantitative phosphoproteomic analysis reveals shared and specific targets of Arabidopsis mitogen-activated protein kinases (MAPKs) MPK3, MPK4, and MPK6**. *Mol Cell Proteomics* 2018, **17**:61–80.

65. ** Rayapuram N, Jarad M, Alhoraibi HM, Bigeard J, Abulfaraj AA, Völz R, Mariappan KG, Almeida-Trapp M, Schlöffel M, Lastrucci E, *et al.*: **Chromatin phosphoproteomics unravels a function for AT-hook motif nuclear localized protein AHL13 in PAMP-triggered immunity**. *Proc Natl Acad Sci Unit States Am* 2021, **118**.
The authors used phosphoproteomics of chromatin-associated protein fractions derived from wild-type *Arabidopsis* and MAPK knockout lines to determine shared and unique MAPK target proteins upon flg22 treatment. They identified the transcription factor AHL13 as a positive regulator of plant immunity and two phosphosites that regulate AHL13 stability *in planta*.

66. Svinkina T, Gu H, Silva JC, Mertins P, Qiao J, Fereshetian S, Jaffe JD, Kuhn E, Udeshi ND, Carr SA: **Deep, quantitative coverage of the lysine acetylome using novel anti-acetyl-lysine antibodies and an optimized proteomic workflow*[S]**. *Mol Cell Proteomics* 2015, **14**:2429–2440.

67. Walley JW, Shen Z, McReynolds MR, Schmelz EA, Briggs SP: **Fungal-induced protein hyperacetylation in maize identified by acetylome profiling**. *Proc Natl Acad Sci USA* 2018, **115**:210.

68. Udeshi ND, Mani DC, Satpathy S, Fereshetian S, Gasser JA, Svinkina T, Olive ME, Ebert BL, Mertins P, Carr SA: **Rapid and deep-scale ubiquitylation profiling for biology and translational research**. *Nat Commun* 2020, **11**:359.

69. Song G, Olatunji D, Montes C, Clark NM, Pu Y, Kelley DR, Walley JW: **Quantitative proteomics reveals extensive lysine ubiquitination in the Arabidopsis root proteome and uncovers novel transcription factor stability states**. *bioRxiv* 2021, https://doi.org/10.1101/2021.01.07.425780.

70. Chen X-L, Xie X, Wu L, Liu C, Zeng L, Zhou X, Luo F, Wang G-L, Liu W: **Proteomic analysis of ubiquitinated proteins in rice (Oryza sativa) after treatment with pathogen-associated molecular pattern (PAMP) elicitors**. *Front Plant Sci* 2018, **9**:1064.

71. Grubb LE, Derbyshire P, Dunning K, Zipfel C, Menke FLH, Monaghan J: **Large-scale identification of ubiquitination sites on membrane-associated proteins in Arabidopsis thaliana seedlings**. *bioRxiv* 2020, https://doi.org/10.1101/2020.09.16.299883.

72. * Ma X, Zhang C, Kim DY, Huang Y, Chatt E, He P, Vierstra RD, Shan L: **Ubiquitylome analysis reveals a central role for the ubiquitin-proteasome system in plant Innate immunity**. *Plant Physiol* 2021, https://doi.org/10.1093/plphys/kiab011.
In contrast to ubiquitin remnant diGly antibody purification, this study used a genetically encoded, tagged ubiquitin and two-step purification

to profile ubiquitinylated protein dynamics within 30 min of flg22 treatment. The authors detected a rapid increase in ubiquitin modifications to the 26S proteasome, ubiquitin ligases, and deubiquitylases, further supporting a central role of these pathways in plant immunity.

73. Cilia M, Johnson R, Sweeney M, DeBlasio SL, Bruce JE, MacCoss MJ, Gray SM: **Evidence for lysine acetylation in the coat protein of a polerovirus**. *J Gen Virol* 2014, **95**:2321.

74. Lv B, Yang Q, Li D, Liang W, Song L: **Proteome-wide analysis of lysine acetylation in the plant pathogen Botrytis cinerea**. *Sci Rep* 2016, **6**:29313.

75. Li D, Lv B, Tan L, Yang Q, Liang W: **Acetylome analysis reveals the involvement of lysine acetylation in diverse biological processes in Phytophthora sojae**. *Sci Rep* 2016, **6**:29897.

76. * McConnell EW, Berg P, Westlake TJ, Wilson KM, Popescu GV, Hicks LM, Popescu SC: **Proteome-wide analysis of cysteine reactivity during effector-triggered immunity**. *Plant Physiol* 2019, **179**:1248.

This study characterized the role of reversible cysteine oxidation during ETI responses in wild-type *Arabidopsis* and the oligopeptidase *top1/top2* mutants with an altered hypersensitive response. Oxidoreductases and defense-related proteins were found to be differentially oxidized in *top1/top2* and temporal patterns of oxidation appeared to track with protein cellular location and biological function.

77. Morimoto K, van der Hoorn RAL: **The increasing impact of activity-based protein profiling in plant science**. *Plant Cell Physiol* 2016, **57**:446–461.

78. Franco JY, Thapa SP, Pang Z, Gurung FB, Liebrand TWH, Stevens DM, Ancona V, Wang N, Coaker G: **Citrus vascular proteomics highlights the role of peroxidases and serine proteases during Huanglongbing disease progression**. *Mol Cell Proteomics* 2020, **19**:1936–1952.

79. Planas-Marquès M, Bernardo-Faura M, Paulus J, Kaschani F, Kaiser M, Valls M, van der Hoorn RAL, Coll NS: **Protease activities triggered by Ralstonia solanacearum infection in susceptible and tolerant tomato lines**. *Mol Cell Proteomics* 2018, **17**:1112–1125.

80. Ilyas M, Hörger AC, Bozkurt TO, van den Burg HA, Kaschani F, Kaiser M, Belhaj K, Smoker M, Joosten MHAJ, Kamoun S, *et al.*: **Functional divergence of two secreted immune proteases of tomato**. *Curr Biol* 2015, **25**:2300–2306.

81. * Paulus JK, Kourelis J, Ramasubramanian S, Homma F, Godson A, Hörger AC, Hong TN, Krahn D, Ossorio Carballo L, Wang S, *et al.*: **Extracellular proteolytic cascade in tomato activates immune protease Rcr3**. *Proc Natl Acad Sci USA* 2020, **117**:17409.

Activity-based protein profiling was used to determine the identity of host proteases that cleave Rcr3 to its active form. The authors determined that a subclass of apoplastic serine proteases called subtilisins activate Rcr3, and this is blocked by the kazal-type protease inhibitor EPI1 from *P. infestans*, thus pointing to a mechanism where pathogens block activation of Rcr3 and other immune-related cysteine proteases by inhibiting their upstream activators.

82. Shindo T, Kaschani F, Yang F, Kovács J, Tian F, Kourelis J, Hong TN, Colby T, Shabab M, Chawla R, *et al.*: **Screen of non-annotated small secreted proteins of Pseudomonas syringae reveals a virulence factor that inhibits tomato immune proteases**. *PLoS Pathog* 2016, **12**:e1005874.

83. ** Buscaill P, Chandrasekar B, Sanguankiattichai N, Kourelis J, Kaschani F, Thomas EL, Morimoto K, Kaiser M, Preston GM, Ichinose Y, *et al.*: **Glycosidase and glycan polymorphism control hydrolytic release of immunogenic flagellin peptides**. *Science* 2019, **364**:eaav0748.

This study used activity-based profiling to identify hydrolases in the apoplast that are inhibited by *P. syringae* during infection, ultimately identifying an important role for β-galactosidase 1 in deglycosylating bacterial flagellin, thus exposing it to host proteases that process it into immunogenic flg22 peptides that are recognized by plants.

84. Salguero-Linares J, Coll NS: **Plant proteases in the control of the hypersensitive response**. *J Exp Bot* 2019, **70**:2087–2095.

85. Balakireva AV, Zamyatnin AA: **Indispensable role of proteases in plant innate immunity**. *Int J Mol Sci* 2018, **19**.

86. Hou S, Jamieson P, He P: **The cloak, dagger, and shield: proteases in plant–pathogen interactions**. *Biochem J* 2018, **475**:2491–2509.

87. Kim SH, Qi D, Ashfield T, Helm M, Innes RW: **Using decoys to expand the recognition specificity of a plant disease resistance protein**. *Science* 2016, **351**:684.

88. Carter ME, Helm M, Chapman AVE, Wan E, Restrepo Sierra AM, Innes RW, Bogdanove AJ, Wise RP: **Convergent evolution of effector protease recognition by Arabidopsis and barley**. *MPMI (Mol Plant-Microbe Interact)* 2018, **32**:550–565.

89. Helm M, Qi M, Sarkar S, Yu H, Whitham SA, Innes RW: **Engineering a decoy substrate in soybean to enable recognition of the soybean mosaic virus Nla protease**. *MPMI (Mol Plant-Microbe Interact)* 2019, **32**:760–769.

90. Kleifeld O, Doucet A, auf dem Keller U, Prudova A, Schilling O, Kainthan RK, Starr AE, Foster LJ, Kizhakkedathu JN, Overall CM: **Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products**. *Nat Biotechnol* 2010, **28**:281–288.

91. Gevaert K, Goethals M, Martens L, Van Damme J, Staes A, Thomas GR, Vandekerckhove J: **Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides**. *Nat Biotechnol* 2003, **21**:566–569.

92. Venne AS, Solari FA, Faden F, Paretti T, Dissmeyer N, Zahedi RP: **An improved workflow for quantitative N-terminal charge-based fractional diagonal chromatography (ChaFRADIC) to study proteolytic events in Arabidopsis thaliana**. *Proteomics* 2015, **15**:2458–2469.

93. Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, Dinesh-Kumar SP: **MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays**. *Genes Dev* 2009, **23**:80–92.

94. Jayaraman D, Richards AL, Westphall MS, Coon JJ, Ané J-M: **Identification of the phosphorylation targets of symbiotic receptor-like kinases using a high-throughput multiplexed assay for kinase specificity**. *Plant J* 2017, **90**:1196–1207.

95. Wang P, Hsu C-C, Du Y, Zhu P, Zhao C, Fu X, Zhang C, Paez JS, Macho AP, Tao WA, *et al.*: **Mapping proteome-wide targets of protein kinases in plant stress responses**. *Proc Natl Acad Sci Unit States Am* 2020, **117**:3270–3280.

96. Adams JA: **Activation loop phosphorylation and catalysis in protein Kinases: is there functional evidence for the autoinhibitor model?** *Biochemistry* 2003, **42**:601–607.

97. Ahiri A, Garmes H, Podlipnik C, Aboulmouhajir A: **Insights into evolutionary interaction patterns of the "Phosphorylation Activation Segment" in kinase**. *Bioinformation* 2019, **15**:666–677.

98. Walley JW, Shen Z, Sartor R, Wu KJ, Osborn J, Smith LG, Briggs SP: **Reconstruction of protein networks from an atlas of maize seed proteotypes**. *Proc Natl Acad Sci Unit States Am* 2013, **110**:E4808–E4817.

99. Beekhof R, van Alphen C, Henneman AA, Knol JC, Pham TV, Rolfs F, Labots M, Henneberry E, Le Large TY, de Haas RR, *et al.*: **INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases**. *Mol Syst Biol* 2019, **15**:e8981.

100. Schmidlin T, Debets DO, van Gelder CAGH, Stecker KE, Rontogianni S, van den Eshof BL, Kemper K, Lips EH, van den Biggelaar M, Peeper DS, *et al.*: **High-throughput assessment of kinome-wide activation states**. *cell Syst* 2019, **9**:366–374.e5.

101. Mishra B, Kumar N, Mukhtar MS: **Systems biology and machine learning in plant–pathogen interactions**. *MPMI (Mol Plant-Microbe Interact)* 2018, **32**:45–55.

102. Peyraud R, Dubiella U, Barbacci A, Genin S, Raffaele S, Roby D: **Advances on plant–pathogen interactions from molecular toward systems biology perspectives**. *Plant J* 2017, **90**:720–737.

103. Eicher T, Kinnebrew G, Patt A, Spencer K, Ying K, Ma Q, Machiraju R, Mathé EA: **Metabolomics and multi-omics integration: a survey of computational methods and resources**. *Metabolites* 2020, **10**.

104. Walley JW, Sartor RC, Shen Z, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR, *et al.*: **Integration of omic networks in a developmental atlas of maize**. *Science* 2016, **353**:814–818.

105. Vitrinel B, Koh HWL, Mujgan kar F, Maity S, Rendleman J, Choi H, Vogel C: **Exploiting interdata relationships in next-generation proteomics analysis**. *Mol Cell Proteomics* 2019, **18**: S5–S14.

106. * Delplace F, Huard-Chauveau C, Dubiella U, Khafif M, Alvarez E, Langin G, Roux F, Peyraud R, Roby D: **Robustness of plant quantitative disease resistance is provided by a decentralized immune network**. *Proc Natl Acad Sci USA* 2020, **117**:18099.
The authors integrated transcriptomics, microscopy, protein-protein interactome data using network analysis to understand how the atypical kinase RKS1 controls quantitative disease resistance (QDR) in *Arabidopsis.* They found that the RKS1 signaling network is highly connected and distributed across different subcellular locations and subsequent mutant analyses revealed specific subnetworks with dominant roles in QDR.

107. Ding Y, Murphy KM, Poretsky E, Mafu S, Yang B, Char SN, Christensen SA, Saldivar E, Wu M, Wang Q, *et al.*: **Multiple genes recruited from hormone pathways partition maize diterpenoid defences**. *Nat Plants* 2019, **5**:1043–1056.

108. Ramsey JS, Chin EL, Chavez JD, Saha S, Mischuk D, Mahoney J, Mohr J, Robison FM, Mitrovic E, Xu Y, *et al.*: **Longitudinal transcriptomic, proteomic, and metabolomic analysis of citrus limon response to graft inoculation by Candidatus liberibacter asiaticus**. *J Proteome Res* 2020, **19**: 2247–2263.

109. Chin EL, Ramsey JS, Mishchuk DO, Saha S, Foster E, Chavez JD, Howe K, Zhong X, Polek M, Godfrey KE, *et al.*: **Longitudinal transcriptomic, proteomic, and metabolomic analyses of citrus sinensis (L.) Osbeck graft-inoculated with "Candidatus liberibacter asiaticus."** *J Proteome Res* 2020, **19**: 719–732.

110. * Sekiya A, Marques FG, Leite TF, Cataldi TR, de Moraes FE, Pinheiro ALM, Labate MTV, Labate CA: **Network analysis combining proteomics and metabolomics reveals new insights into early responses of Eucalyptus grandis during rust infection**. *Front Plant Sci* 2021, **11**:2165.
This study in a nonmodel plant integrated proteome and metabolome analysis using network analysis to correlate protein modules with metabolites during rust infection. Phenylpropanoid metabolism was differentially affected in temporal waves during the first 24 h of infection, and the authors predicted the proteins associated with these dynamics.

111. * Nobori T, Wang Y, Wu J, Stolze SC, Tsuda Y, Finkemeier I, Nakagami H, Tsuda K: **Multidimensional gene regulatory landscape of a bacterial pathogen in plants**. *Nat Plants* 2020, **6**:883–896.
Proteome and transcriptome profiling was performed on bacteria populations isolated from infected wild-type and salicylic acid (SA) biosynthetic and signaling mutants. They found an overall correspondence in mRNA and protein regulation, but specific processes such as chemotaxis and translation were differentially impacted at the protein level in response to host SA signaling.