Chapter 9
Plant Response to Bacterial Pathogens: A Proteomics View

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Abstract Plants rely on their innate immunity comprised of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) for defense against pathogens. The evolution of this immune response has resulted in a highly effective system of defense that is able to resist potential attack by pathogens. Bacterial pathogens are major threats to crop production and result in vast losses in revenue each year. Thus, better understanding of the interactions between plants and pathogenic bacteria is a promising avenue for the improvement of crop productivity and agriculture sustainability. Proteomic technologies provide a unique angle to study the intricate interactions between plants and pathogens. Approaches for proteomic analysis can not only lead to the identification of proteins, but also provide quantification and characterization of post-translational modification (PTM). Here we highlight the current knowledge of plant innate immunity in response to bacterial pathogens. We also discuss interesting plant proteomic responses, as well as address the exciting areas of secretome and PTM proteomics as they closely relate to plant-bacteria interactions.

Keywords Proteomics · Bacterial pathogen · Secretome · Post-translational modification

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9.1 Introduction

Plants are in constant interaction with microbes. Most interactions are not harmful, however, those with pathogenic microbes can lead to diseases that affect plants ability to thrive and reproduce. The diseases caused by the phytopathogens are widespread and often results in a significant decrease in crop yield and economic loss, thus threatening the global food security. It is estimated that over 10 % of the world's agriculture crops are lost to plant-pathogens annually with much greater losses occur during times of epidemics [1]. As the world's population continues to grow, land available for farming decreases and pathogens continue to evolve resistance to plant defenses, better understanding of plant-pathogen interactions is critical to the development of more useful strategies of plant protection against pathogens and increase crop productivity and global food security [2]. Among the many types of phytopathogens, which include fungi, viruses and oomycetes, disease causing bacteria are major threats to crop production [3] and will be the focus of this review.

Bacteria are single-celled prokaryotic organisms found in vast numbers in nearly every place on Earth. They are classified into two major groups, Gram negative and gram positive, based on their chemical composition and cell wall structure [4]. The interactions between bacteria and plants are often beneficial for the plants and/or the bacteria. Such is the case for nitrogen-fixing bacteria in the roots of certain legumes and other plant growth-promoting bacteria as well as those involved in the decomposition of plant remains [5]. Beneficial interactions are common and pose agronomic importance, and plant microbiome research has started to gain a lot of attraction [6]. However, in many cases bacteria can be harmful to plants causing disease and result in major economic loss and increase famine due to decrease in crop production [1]. Most disease causing plant bacterial pathogens belong to the following genera: *Pantoea, Burkholderia, Acidovorax, Clavibacter, Streptomyces, Spiroplasma, and Phytoplasma, Pseudomonas, Ralstonia, Xanthomona, Pectobacterium, Agrobacterium, Xylella, Erwinia, Dickeya* [7]. The top ten scientific and economically important bacterial pathogens fall in the genera of the latter eight and cause diseases such as bacterial speck of tomato, potato brown rot, leaf blight of rice, and fire blight of apple and others that lead to significant economic loss of agriculture crops [8]. These pathogens often use sophisticated molecular strategies to cause plant disease, and plants must overcome them in a biological race to compete and survive.

Currently, an environmental friendly method of battling diseases of agriculture crops involves the development of crop cultivars with enhanced resistance to those pathogenic bacteria that normally cause disease [9]. This requires knowledge of molecular interactions involved in promoting or suppressing disease. Given that proteins play a large role in the interactions between plant and pathogen, proteomics is a logical tool for investigating and elucidating molecular mechanisms that lead to disease. In recent years there has been significant progress made to better understanding plant-bacterial interactions with the use of proteomics technologies and

approaches. The use of these approaches has enabled the identification of and changes in proteins involved in plant-bacteria communication during infection. Here we first highlight the current knowledge of plant innate immunity in response to bacterial challenge. Next, we discuss plant proteomic responses to bacteria pathogens with special attention to agriculture crops. Lastly, we address the expanding areas of secretome and post-translational modification (PTM) proteomics as they closely relate to plant-pathogen interactions.

9.2 Current Knowledge of Plant Innate Immune Response to Bacteria

9.2.1 Pathogen Associated Molecular Patterns (PAMPs)

The interaction between plant and bacterial pathogens involves a multifaceted process mediated by both plant and pathogen derived molecules that included proteins, sugars and lipopolysaccharides [10]. To cause disease, pathogens must successfully enter and colonize inside the host. The cell wall and cuticle of plants act as natural physical barriers to prevent the invasion of pathogens. However, these natural defenses are not always effective at preventing invading pathogens. Successful resistance comes from the plants dual level innate immune system of active defense responses [11]. The first level of plant active defense involves the recognition of microbial molecules or elicitors by the plant. These molecules or elicitors are known as pathogen-associated molecular patterns (PAMPs) and are evolutionarily conserved components of microbes/pathogens [12]. Research throughout the years has generated a list of known PAMPs from various bacteria and their induced responses. Common examples of PAMPs include flagellin, elongation factor Tu (EF-Tu), cold shock proteins (CPS), and lipopolysaccharides (LPS) and are all characteristic of gram negative bacteria [13]. Although not all PAMPs are identical across bacteria, conserved protein regions have been shown to provide an avenue for studying PAMP response mechanisms in plants. The most extensively studied PAMP is the small peptide flg22 derived from bacterial flagellin. Flagellin is a protein component of flagella, the structure that provides mobility to certain cells including many gram negative bacteria. Both the N and C terminal regions of this protein are evolutionarily conserved across a number of bacterial species [14], making flagellin useful when studying plant defense against bacterial pathogens. In an early study, purified flagella from *P. tabaci* was shown to initiate downstream responses to the bacterial PAMP in both tomato and Arabidopsis [15]. Early work provided multiple examples of pathogens capable of evading the plant recognition system due to mutations within flg22 epitope, which further demonstrated the importance of flagellin in plant-bacterial interactions [15–17]. In addition to flagellin, several other PAMPs (e.g., EF-Tu and LPS) have been identified and shown to induce downstream responses to bacterial PAMPs in different systems [18–22].

9.2.2 PAMP Triggered Immunity (PTI)

In response to PAMPs, plants have evolved mechanisms to detect conserved bacteria components. In plants, PAMPs are recognized by pattern recognition receptors (PRRs). These receptors are localized on the plant cell membrane and are either leucine-rich receptor-like kinases (LRR-RLK) or receptor-like proteins (RLP) [23]. Like PAMPs, several PPRs have been characterized and include Flagellin Sensitive 2 (FLS2), the flagellin receptor and EF-Tu receptor (EFR), the bacterial elongation factor EF-Tu receptor. Recognition of PAMPs by plant PRRs leads to the activation of the first level of plant innate immune response (Fig. 9.1). This PAMP activated immune response and subsequent changes within the plant are often referred to as basal defense response, general elicitor response and PAMP triggered immunity (PTI), each describing the same level of response that PAMP recognition induces within the plant. This form of immunity is characterized by an assortment of molecular and genetic changes, which include alkalinization, changes in ion flux, increase in reactive oxygen species (ROS), and activation of mitogen-activated protein kinase (MAPK) cascades (Fig. 9.1) [24–26]. PTI can be considered a

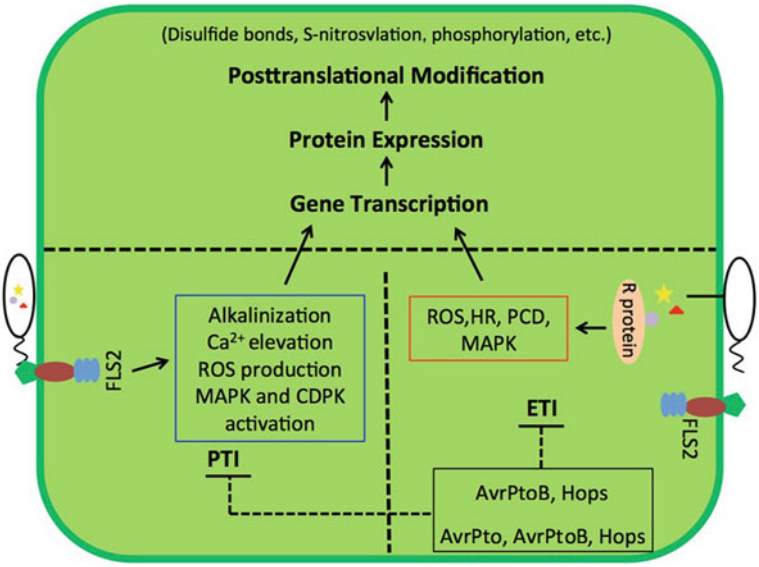

Fig. 9.1 Diagram showing interaction between plants and pathogen infection. Plants respond to PAMPs such as flg22 through PRRs and induce PTI mechanisms including alkylation, Ca^{2+} elevation, ROS production, MAPK, and CDPKs activation. These response mechanisms are inhibited by T3Es such as AvrPto, AvrPtoB, and Hops. ETI response mechanisms are activated through identification of T3Es and production of ROS, HR, PCD, and activation of MAPKs. ETI is also regulated through T3Es such as AvrPtoB and Hops. Both early and late response mechanisms have been shown to initiate changes in gene transcription, translation and PTMs such as disulfide bond formation, S-nitrosylation and phosphorylation

"priming" mechanism of plants defense response to bacteria, so that if a plant is challenged by a secondary infection after initial PTI has been allowed to develop, bacterial growth is often restricted in host plants and eliminated in non-host plants [27]. Over the last decade, there has been much research that has led to our understanding of PTI signaling cascades. Research here has involved mutations of known receptors such as FLS2 and EFR in order to examine phenotype of PRR mutants as well as elucidate signaling components of PAMP induced PTI and pathogen development [19, 27–29].

9.2.3 Effector Triggered Immunity (ETI)

As previously described, PTI is a basal defense response utilized by the plant to prevent bacterial growth. In order to successfully infiltrate the apoplast and thrive, bacteria must bypass this basal defense. Bacteria have therefore evolved mechanisms to overcome different components of PTI by delivering 'effector' proteins into the plant. A mechanism in which many pathogenic bacteria such as certain species from the genera *Pseudomonas, Ralstonia, Xanthomona, Erwinia* accomplish delivery of effectors is through the use of a type III secretion system (TTSS). Utilizing this system, bacteria are able to bypass the plant cell wall and plasma membrane to inject effectors directly into the cell (Fig. 9.1) and successfully suppress PTI. Plants are often able to perceive this and mount a subsequent response. The second level of plant innate immune response involves the recognition and interaction of these specific effectors known as avirulence (Avr) proteins or type three effectors (T3E) by resistance proteins (R proteins) within the plant and is referred to as effector-triggered immunity (ETI). An adaptive zig-zag model has been proposed and is representative of the changes in the infection and defense processes [11]. Successful recognition of avr proteins, like AvrPto, AvrPtoB, and Hops from *Pseudomonas syringae* by plant R proteins leads to a localized cell death (hypersensitive response), the expression of pathogenesis related (PR) genes, changes in the proteome and systemic acquired resistance (SAR) in order to kill the pathogen, pathogen infected cells and help prevent later infections [30, 31]. A number of studies have highlighted the role and provided support to the importance of several factors involved in ETI [32–35]. Although ETI response is described as being faster and more robust when compared to PTI, both PTI and ETI act together to effectively defend plants against pathogens and common to them both is an increase in reactive oxygen species (ROS), production of nitric oxide (NO), and changes in PTM events in order to prevent pathogen infection or inhibition of its growth.

9.3 Proteomic Responses to Bacterial Pathogens

Despite the knowledge of plant innate immunity, many components of PTI and ETI in response to bacteria pathogens remain largely unknown. As previously stated, proteins play an undeniable role in plant-pathogen interactions, and proteomics has become a valuable resource for better understanding these interactions. It is known that information obtained from genomics and transcriptomics does not always correlate to protein changes [36]. Providing insights to protein localization, enzymatic complexes, protein-protein interactions and PTMs, proteomics allow a more direct view of cellular processes and activities that occur during specific plant-pathogen interactions. Advances in proteomic technologies such as two-dimensional gel electrophoresis (2-DE), two-dimensional difference gel electrophoresis (2D-DIGE), and gel free methods such as isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), cysteine reactive tandem mass tags (cysTMT), and stable isotope labeling by amino acids in cell culture (SILAC) in tandem with liquid chromatography (LC)-mass spectrometry (MS) have made it possible for the large scale analysis of proteomic changes during plant-pathogen interactions under various treatment conditions and times (Fig. 9.2). Results of proteomic work aid in filling knowledge gaps that arose during the genomic and transcriptomic area. With the great improvements in these technologies within the past 20 years there has been an increase in the amount proteomic literature in the area of plant-bacteria interactions (Table 9.1). Much of the research here highlights changes in protein modification and expression levels induced by treatment with bacterial pathogens or elicitors and relies on the use of MS approaches to provide qualitative and quantitative information. In the area of plant-bacteria proteomics, the symbiotic interaction between nitrogen fixing bacteria and legumes has long been the most well studied [37]. In more recent years, however, research has been increasingly focused on the proteomes of plant during their interactions with bacterial pathogens.

9.3.1 General Proteomic Studies

A number of proteomics studies using non-crop model systems have contributed to what is known regarding plant–pathogen interactions. An important system is that of the model plant *Arabidopsis thaliana*, which has been invaluable to the understanding of molecular events leading to the progression of disease or successful pathogen defense in crops [38]. Arabidopsis along with bacterial pathogens and bacterial elicitors has been used to identify specific proteins that respond to bacterial pathogens. Using bacterial elicitors rather than the pathogen itself to treat the host plants or cell suspension cultures simplifies the system, making it easier to study rapid changing in the proteome. Therefore, some plant defense studies have utilized Flg22, chitin or other bacterial elicitors to induce defense responses [39, 40]. However, the

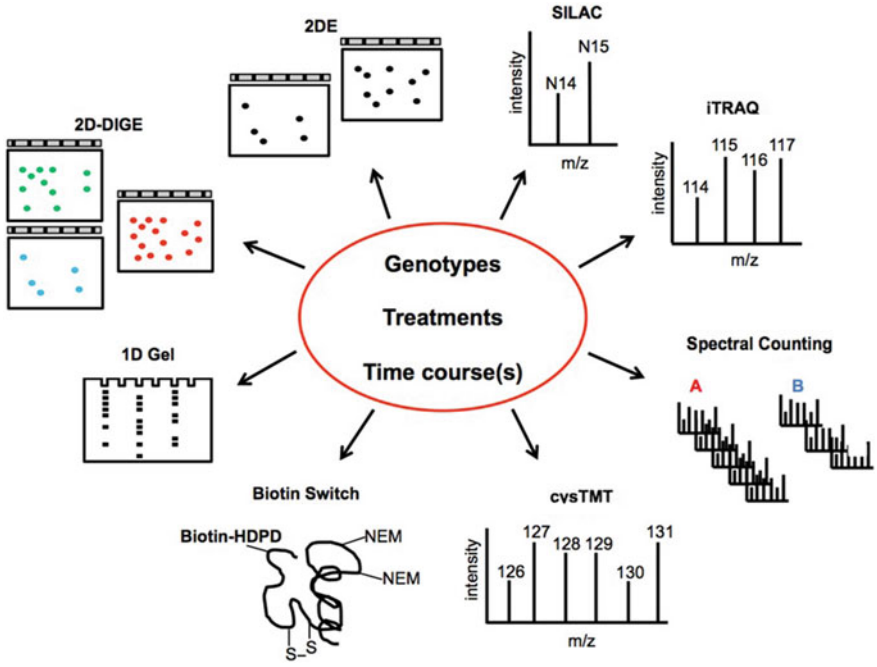

Fig. 9.2 Analytical approaches used in plant pathogen interaction proteomics. 1D SDS-PAGE, 2-DE, and 2DIGE are gel methods used to identify global protein changes and PTMs. SILAC is a gel free method that uses the changes in mass due to the metabolic incorporation of amino acid isotopes to examine quantitative changes between samples. iTRAQ and cysTMT are isobaric tag labeling systems that are used to label protein extracts when examining global protein changes. Labeling with cysTMT and biotin-HDPD is used to identify cysteine redox changes due to different treatment. Spectral counting is a label-free method capable of examining changes in protein levels and PTMs

use of bacterial pathogens themselves provides a more complete system and often reveals more information about the interactions and will be the focus here.

As a lab model system, the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 has often been used to study plant response to bacteria pathogens. *Pst* is a gram negative bacterium that causes bacterial speck disease in *A. thaliana* and *Solanum lycopersicum* (tomato). Because of the necrotic lesion that forms on the plant after infection, plants and their fruits become less valuable. Jones and colleagues analyzed PAMP, TTSS, and ETI proteomic changes in Arabidopsis when challenged with different near isogenic lines of (*Pst*) DC3000 [41, 42]. The study reported defense-related PR-9 antioxidant proteins and metabolic enzymes. Two groups of proteins (glutathione S-transferase (GST) and peroxiredoxin (Prx) changed in response to treatment with *Pst* and its variants, *hrpA* DC3000 and *Pst* (*avrRPM1*) [41]. These two groups of antioxidant enzymes are suggested to play important roles in the regulation of redox conditions during pathogen infection. Further studies using early time points, protein extracts from the chloroplast,

Table 9.1 Proteomic studies of plant response to bacterial pathogens

Organism	Pathogen/elicitor	Proteomic techniques	Identified proteins	Main findings	References
A. thaliana/suspension cells	Flg22 or chitin fragments	2-DE, nESI-MS/MS	AtPhos43, and related proteins	AtPhos43 phosphorylation is rapid and dependent on FLS2	[39]
A. thaliana/suspension-cultured cells	Flg22	Labelling with 32P, SAX-IMAC, MALDI MS, nanoLC-MS/MS	299 phosphopeptides; ~200 phosphoproteins	Large-scale ID of phosphorylation sites; previously unknown phosphorylation sites on proton ATPase isoforms	[40, 67]
A. thaliana/leaves	*Pseudomonas syringae* pv. *tomato* DC3000, DC3000 *avrRpm1*, DC3000 *hrpA*	2-DE, LC-MS/MS	53 proteins, two groups GSTs and Prxs analyzed	glutathione S-transferases and peroxiredoxins, enzyme changes	[41]
A. thaliana/leaves	*P. syringae*	iTRAQTM	5 proteins	Phosphoproteome changes of dehydrin, co-chaperone, HSP, RuBisCO, plastid-associated protein	[42]
A. thaliana/leaves	*P. syringae* pv. *tomato* DC3000, DC3000 *avrRpm1*, DC3000 *hrpA*	2-DE, LC- MS/MS	36 total soluble, 8 chloroplast, 13 mitochondrial proteins	Identified significant changes to defense-related antioxidants and metabolic enzymes involved in ETI and PTI	[43]
A. thaliana/ suspension cells	*P. syringae pv. tomato* DC3000, DC3000 *avrRpm1*, DC3000 *hrpA*	iTRAQ coupled with LC-MS/MS and Q-TOF-MS	45 differentially expressed proteins	Proteins involved in metabolism, redox regulation, defense, and cell wall maintenance	[60]
A. thaliana/ suspension cells	Flg22	iTRAQ, SAX-IMAC, nanoLC-MS/MS	11 proteins	Phosphorylated defense response proteins: NADPH oxidase, RBOHD, and ABC transporter, pleiotropic drug resistance 8, and penetration 3	[67]
A. thaliana/suspension cells	Flg22 or xylanase	$^{4}N/^{15}N$- labelling, SCX-TiO_2, LC-MS/MS	76 proteins, 98 phosphopeptides	Phosphorylated changes in unidentified membrane associated proteins and known defense proteins	[66]

(continued)

Table 9.1 (continued)

Organism	Pathogen/elicitor	Proteomic techniques	Identified proteins	Main findings	References
A. thaliana and *M. sativa*/roots	*P. syringae pv. tomato* DC3000 or *Sinorhizobium meliloti* strain Rm1021	2-DE, nanoLC-MS/MS	~100 secreted proteins	Seven plant proteins, such as hydrolases, peptidases, and peroxidases were increased in *M. sativa–S. meliloti* interaction; Four bacterial proteins increased	[56]
A. thaliana/leaves	*P. syringae pv. tomato* DC3000, DC3000 *AvrB*	2-DE, immunoblotting, MALDI-TOF- TOF	16 proteins	Identification of S-nitrosylated proteins involved in metabolism and defense signaling	[76]
Oryza sativa/transgenic and/or inoculated	*Xanthomonas oryzae* pv. oryzae	2-DE, MS/MS Protein sequencer	10 proteins	Identification of differential expressed proteins related to energy, metabolism, and defense	[51]
O. sativa/suspension cell cultures	*X. oryzae* pv. oryzae	2-DE, MS/MS	20 proteins	Identification of plant proteins involved in early response to bacterial blight	[52]
O. sativa/leaves	*X. campestris* pv. Oryzicola	2-DE, MALDI-TOF MS	32 proteins	Identification of proteins involved in disease resistance signal transduction, pathogenesis, and regulation of cell metabolism	[53]
O. longistaminata/ leaves	*X. oryzae* pv. oryzae	2-DE coupled with MALDI-TOF MS	29 proteins	Proteins for tolerance against the disease: r40c1, cyclin-dependent kinase C, Ent-isokaur-15-ene synthase, glutathione-dependent dehydroascorbate reductase 1 (GSH- DHAR1), and germin-like protein	[54]
O. sativa	*X. oryzae*	2-DE/MudPIT and MALDI-TOF/MS or nESI-LC-MS/MS	109 proteins	Differential enriched proteins involved in pathogenicity, protease/peptidase, and host defense *in planta*, and in vitro	[61]
Lycopersicon hirsutum	*Clavibacter michiganensis* subp. *michiganensis*	2-DE, ESI-MS/MS	47 proteins	Revealed distinct mechanisms conferred by loci Rcm 2.0 and Rcm 5.1, proteins involved in defense, stress responses and cell signaling	[47]

(continued)

Table 9.1 (continued)

Organism	Pathogen/elicitor	Proteomic techniques	Identified proteins	Main findings	References
Solanum lycopersicum	*P. solanacearum*	2-DE, Protein sequence	15 proteins	Proteins involved in protein destination and storage (60 kDa chaperonin, PDI, heat shock protein), protein synthesis, metabolism (e.g., RuBisCO activase, glycine dehydrogenase), and defense (calgranulin, AMA)	[49]
S. lycopersicum	*P. syringae* pv. *tomato* DC3000	iTRAQ, LC-MS/MS	477 proteins	Proteins involved in oxidation-reduction, response to stress, defense signaling, cell wall organization and hormone signaling	[50]
S. lycopersicum	*P. syringae* pv. *tomato* DC3000	CysTMT LC-MS/MS	90 proteins	Potential redox-regulated proteins in carbohydrate/energy metabolism, Cys biosynthesis, sucrose and brassinosteroid, cell wall biogenesis, polysaccharide/starch biosynthesis, cuticle development, lipid metabolism, proteolysis, TCA cycle, protein targeting to vacuole, and oxidation–reduction	[71]
Lotus japonicas	Rhizobial lipochitooligosaccharides nodulation factors (NF)/ Flg22	^{33}P-orthophosphate labeling, 2-DE immunoblotting, kinase assay	13 proteins	Phosphorylation of unique and shared proteins during symbiotic and defense responses	[68]

mitochondria, and total soluble protein and 2-DE and MS/MS, 57 proteins with differences in expression levels were identified [42]. These proteins were determined to be involved in biological processes such as defense (GST), transcription (squamosa promoter binding protein-like 14), protein stability (cyclophilin) and metabolism (glyceraldehydes 3-phosphate dehydrogenase). These results suggest PAMP responses could be attributed to regulation of glycolysis, tricarboxylic acid (TCA) cycle, and antioxidation activities. However, these processes were also altered by the presence of virulent T3E and ETI. PAMP-induced changes at the protein level were reflected by 2D gel spot density changes between mock and *hrpA* DC3000 treatment, a TTSS mutant used to examine PAMP responses and changes induced by T3Es. This revealed a total of 30 proteins identified as having changes in spot density in response to PAMPs. Jones et al. [42, 43] additionally analyzed changes in the phosphoproteome of Arabidopsis during *Pst* infection. Phosphoprotein enrichment followed by iTRAQ tagging was employed and proteins that were differentially phosphorylated in soluble *A. thaliana* leaf extracts were identified. Phosphoproteome changes of 4 proteins (dehydrin, co-chaperone, heat shock protein, plastid-associated protein) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCO LSU) was determined [43]. This first study utilizing iTRAQ to study plant-pathogen interactions highlighted the reproducibility and utility as well as problems often associated with the quantitative analysis of changes in a complex phosphoproteome. In an approach to decrease high abundant proteins in Arabidopsis leaves after *Pst* infection combinatorial hexapeptide ligand libraries (CPLL) were used. The high abundant proteins from infected leaves shown by 2-DE were reduced to a low level and less abundant protein were enriched. Mass spectrometric analysis then led to the identification of 312 bacterial proteins from the infected leaf tissue [44].

The plasma membrane (PM) is where detection of PAMPs occurs and signal transduction is initiated during pathogen response. Using transgenic Arabidopsis with dexamethasone (Dex) inducible AvrRpt2 expression (*GVG-AvrRpt2*), ETI was induced and proteomic changes observed. Using SDS-PAGE and MS/MS, a total of 2336 proteins were identified in PM enriched samples, with 423 proteins showing significant changes in expression [45]. A total of 235 proteins with increased levels were involved in processes such as camalexin biosynthesis (CYP71B15, CYP71A12, CYP71A13), membrane trafficking (syntaxin of plants (SYP) 122, soluble N-ethylmaleimide-sensitive factor adaptor protein 33), protein phosphorylation (Pep1 receptor 1, wall-associated kinase 1, cysteine-rich receptor kinase), and methionine metabolism (S-adenosylmethionine synthase, 1-aminocyclopropane-1-carboxylate oxidase). Proteins with decrease levels, a total of 188, were involved in biological processes that included glucosinolate metabolism (e.g., CYP83A1 and CYP83B1), membrane transport (auxin and Ca^{2+} transporters), and photosynthesis (thylakoid membrane proteins). This information provided insight into the specific ETI regulated changes occurring at the PM. Virulent *Pst* and avirulent *Pst* (avrRpt2) treatment of Arabidopsis was used to investigate the function of time in the development of the response mechanisms [46]. Utilizing 2-DE and MS/MS, the authors identified 800 proteins, with 147 showing significant changes. After the virulent

infection, 794 spots were present at 4 hai, 795 present at 8 hai, and 772 present at 24 hai. Avirulent infection resulted in observation of 808 spots present at 4 hai, 810 at 8 hai, and 739 at 24 hai. Proteins were categorized as being shared between the virulent and avirulent response mechanism, or unique to one or the other response. A total of 23 proteins that were not previously identified as being involved in defense were identified, e.g., RAS GTP-binding nuclear protein and NUDIX (nucleoside diphosphates linked to moiety X) hydrolase homolog. Results of this study provide information on proteomic changes occurring not only between virulent and avirulent responses, but also provide a snapshot of the proteomic changes occurring at different time points during the infection.

Although proteomic research using Arabidopsis is extensive and has improved what is known about plant-pathogen communication (additional studies with the model plant will be described in other sections), studies using other systems and various pathogens has been helpful in studying specific plant responses to bacterial infection. Proteomic work utilizing the wild tomato species *L. hirsutum* revealed proteins that are regulated in response to *Clavibacter michiganensis* subp. *michiganensis* infection. This bacterial pathogen is responsible for bacterial canker disease in tomato, leading to leaf necrosis, leaf wilt, cankers on the stem and plant death. Two partially resistant lines that contained the quantitative trait loci Rcm2.0 and Rcm 5.1 which control resistance to the disease and a susceptible line were compared using 2-DE and MS [47]. Analysis identified 26 differentially regulated proteins, of which three superoxide dismutase (SOD) enzymes and nine other enzymes directly related to plant defense were identified. The identification of these enzymes indicates the importance of stress related proteins including those related to oxidative stress in response to pathogenic infection and revealed distinct mechanisms conferred by two loci: Rcm 2.0 and Rcm 5.1. In a study to detect genes that control tomato (*L. esculentum*) bacterial wilt infected with *Ralstonia solanacearum* [48] reported that expression of the caffeoyl coenzyme A (CoA) 3-O-methyltransferase gene was down-regulated in seedlings of different susceptible cultivars of tomato that were inoculated with the bacteria. The new discovery of this down-regulated gene in infected tomato suggests its role in tomato response to stress such as that caused by bacterial infection. Further, proteomic research in the tomato system reported the differential levels of proteins in response to the necrotrophic bacteria *Pseudomonas solanacearum* [49]. Using 2-DE and Edman sequencing, the comparison of protein fold changes between bacterial wit-sensitive and wit-resistant cultivars revealed nine proteins that were highly expressed in resistant cultivars. The proteins were found to be related to plant defense, protein storage and protein trafficking. As well, the apical membrane antigen (AMA) was found to be increased in the susceptible cultivars. This provided support that a previously unidentified protein has a role in tomato resistance to this bacteria pathogen. Additionally, Parker and colleagues recently examined plant pathogen interactions between tomato and *Pst* DC3000 and identified 2369 proteins amongst two genotypes (*PtoR* and *prf3*) and two time points (4 hai and 24 hai) [50]. Of the proteins, 477 showed significant changes in levels. Proteins with significant changes in *PtoR* 4 hai (225) included proteins involved in cell wall

organization and reduction of oxidative stress. *PtoR* 24 hai had the most proteins (325) with significant changes when compared amongst genotypes and time points. Those proteins were identified as being involved in oxidation-reduction, response to stress, and signaling. When 164 proteins identified in *prf3* 4 hai were examined, it was observed that although there were fewer proteins identified in this genotype and treatment when compared to both time points in *PtoR*, more proteins were identified when compared to *prf3* 24 hai. It was also noted that *prf3* 4 hai and *PtoR* 24 hai had shared biological processes such as immune response, response to biotic stimulus, and hormone metabolic process, suggesting defense response processes may be induced early after infection of susceptible genotypes, but repressed in *prf3* 24 hai. Biological processes associated with abiotic stress and oxidation-reduction were observed in *prf3* 24 hai. This genotype and time point had the fewest number of proteins (128) with significant changes in levels, but the most proteins with a decrease in expression that paralleled the decreased defense response and onset of the diseased phenotype.

Additional proteomic studies employed other plant species as pathosystems to studying plant response to pathogens. In rice bacterial blight, caused by *Xanthomonas oryzae* pv. oryzae (Xoo) is one of the most severe diseases of this crop, leading to major crop loss during server epidemics. In work by Mahmood and colleagues [51], the defense-related antioxidants PR-9 and PR-5 in rice were determined to be induced in response to treatment with Xoo. Analysis revealed four defense-related proteins PR-5, Probenazole-inducible protein (PBZ1), SOD and Prx were induced for both compatible and incompatible *X. oryzae* pv. Rapid induction and higher expression of PR-5 proteins and PBZ1 were seen in incompatible interactions and in the presence of the plant hormone jasmonic acid (JA). As well the work here presented PR-5 (Thaumatin like protein) as a candidate to use in plant biotechnology against bacterial blight in rice. In an effort to study the early defense responses involved in the rice receptor kinase Xa21 mediated resistance proteins from rice, plasma membrane from suspension cell cultures inoculated with both compatible and incompatible Xoo race strains were analyzed [52]. Twenty proteins were found differentially regulated in cultures that were induced by the pathogen at 12 and 24 h post-inoculation. Of the twenty proteins that have potential function in rice defense, eight were plasma membrane associated and two were non plasma membrane associated.

Li and colleagues identified proteins responsive to *X. campestris* pv. *Oryzicola,* the causative agent of bacterial leaf streak (BLS) another major disease in rice. Using 2-DE coupled with matrix assisted laser desorption ionization (MALDI)-time of flight (TOF) MS analysis, 32 increased proteins that are potentially involved in disease resistance signal transduction, pathogenesis, and regulation of cell metabolism were identified. In addition, seven gene transcripts were shown to be increased after bacterial infection [53]. This helped to elucidate the molecular mechanisms underlying BLS disease. Recently in a comparative proteomic analysis of total foliar protein isolated from infected rice leaves of susceptible Pusa Basmati 1 (PB1) and resistant *Oryza longistaminata* genotypes, proteins belonging to a large number of biological and molecular functions potentially involved in *Xoo* infection

as well as candidate genes conferring tolerance against bacterial blight were identified [54]. Using 2-DE coupled with MALDI-TOF MS, 29 protein spots encoding unique proteins from both the genotypes were identified. Among the proteins those related to biotic and abiotic stress were induce during infection, which suggests that both pathways are activated during infection. The identified candidate genes for tolerance against the disease include putative r40c1, cyclin-dependent kinase C, Ent-isokaur-15-ene synthase and glutathione-dependent dehydroascorbate reductase 1 (GSH-DHAR1), which were induced, and the germin-like protein which was induced only in the resistant genotype.

9.3.2 Secretome Studies

Pathogenesis depends on the ability of the pathogen to manipulate the plant metabolism and to inhibit plant immunity, which depends to a large degree on the plant's capacity to recognize pathogen elicitors. The first interaction between plant and pathogens occurs in the apoplast, thus analyzing the changes of apoplastic proteins through a proteomics approach is important to the understanding of the components of signal perception and signal transduction during pathogen attack. The extracellular secreted proteins in the apoplast at a given time are known as the secretome. Despite its importance, the secretome during plant-bacteria interactions remains poorly characterized compared to the intracellular proteome [38]. The dearth of literature in this area most likely results from the difficulty involved in obtaining apoplastic material without damaging the plant cell and the lack of better methods of preventing contamination of cytoplasmic proteins [55]. In a study of the root secretomes during the interaction between *Medicago sativa* (alfalfa) and the bacterial symbiont *Sinorhizobium meliloti* and between *A. thaliana* and the bacterial pathogen *P. syringae*, bacteria were shown to change the proteins they secreted during infection depending on the identity of the host plant [56]. Using a vacuum infiltration (VIC) method to isolate the apoplastic proteins from the root exudates, more than 100 proteins were identified as differentially expressed during the different plant-bacteria interactions. Of the identified proteins seven plant proteins, such as hydrolases, peptidases, and peroxidases were increased in *M. sativa-S. meliloti* interaction. In addition, four bacterial proteins increased during *S. meliloti/alfalfa* interaction and nine plant defense-related proteins increased during *P. syringae* DC3000/Arabidopsis compatible interaction. This study uncovered a specific, protein level cross-talk between roots and two bacteria pathogens. Several studies have used suspension cell cultures in secretome research [57–59]. Secreted proteins can be easily separated from suspended cells in suspension cell cultures without disrupting the cell, thus preventing potential contamination by cytoplasmic proteins. In an experiment to examine proteomic changes in the secretome after pathogen infection, *A. thaliana* suspension cell cultures were treated with different strains of *Pst.* Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and iTRAQ, the researchers identified changes in 45 proteins, which

include glucan endo-1,3-β-glucosidase 7, Prx 53, strictosidine synthase, and EF-2 [60]. Nine proteins, that contained signaling peptides, were observed to be commonly suppressed after treatment with each strain, suggesting that their levels may be regulated by PAMP detection and response mechanisms. Additionally, eight proteins were identified as regulated specifically by T3Es. Gene for gene resistance induced by *Pst* (*avrRpm1*) caused accumulation of 12 proteins. The role that T3Es and gene for gene resistance play in the regulation of proteins induced by PAMP response was also examined. *Pst* (*hrpA*$^{-}$) induced three proteins, and one was suppressed by T3Es. However, one protein induced by PAMP was suppressed by T3Es, but the levels increased again after gene for gene resistance was introduced through *Pst* (*avrRPM1*). A total of six proteins without a signaling peptide were suppressed by PAMP treatment. The same proteins were identified as being induced after T3E treatment and had reduced levels after gene for gene resistance was induced. Regulation of protein levels by T3Es suggests a role in successful pathogen infection.

Apoplastic proteins were also analyzed during bacterial infection of rice [61]. Analysis of Xoo (compatible race K3)-secreted proteins, isolated from its in vitro culture and *in planta* infected rice leaves using 2-DE coupled with MS, 109 proteins were identified. Only six of the identified proteins were secreted from rice, indicating that the percentage of bacterial-secreted proteins is much higher than its host rice. The identified proteins secreted from *X. oryzae* in vitro and *in planta*, belonged to multiple biological and molecular functions. Proteins involved in metabolic and nutrient uptake activities were common to both in vitro and *in planta* secretomes. However proteins involved in pathogenicity, protease/peptidase, and host defense were highly enriched *in planta*, but not detected in vitro. Information obtained from this work furthers the knowledge of rice bacteria blight disease.

9.3.3 PTM Proteomics

PTMs are known to control many physiological processes by affecting protein structure, activity, and stability. To date hundreds of PTMs have been described, however, only a few have been analyzed using large-scale proteomic techniques. Proteins can undergo different PTMs such as phosphorylation and redox-based regulation such as nitrosylation. Constitutive activation of defense response mechanisms often results in reduced plant growth and yield. The ability to turn on and off a "molecular switch" through PTMs such as phosphorylation and nitrosylation allows plants to respond quickly and efficiently to environmental challenges (Fig. 9.1). It should be noted that pathogens also have the ability to modify proteins. Modifications to the proteins involved in plant-pathogen interaction often suppress or initiate their activities [62]. For example, it is known that AvrPto-induced HR is mediated through autophosphorylation of Pto and that mutations rendering the protein incapable of autophosphorylation results in decreased ETI [63]. It is also known that phosphorylation on T199A of Pto P+1

loop is required for the interaction with Pto and AvrPtoB to occur [30]. In addition, PTMs can take place on T3Es themselves, thus affecting their ability to modulate plant defense responses. For example, AvrPto is phosphorylated by a 30-40 kDa kinase on serine 149 (S149). Treatment of *prf3* with the S149A mutant resulted in decreased bacterial growth. Also, treatment of PtoR with S149A showed a decrease in bacteria, showing that S149A is important for the avirulence responses initiated by ETI [64]. HopAI1 is another example of T3E capable of direct T3E-triggered modifications. In Arabidopsis, this protein interacts with MPK3/MPK6 and through de-phosphorylation inactivates the kinases involved in PTI and leads to dampening of PTI activated MAPK signaling cascades [65].

The study of phosphorylation in early response to flg22 and xylanase has been performed in Arabidopsis [66]. Phosphorylated peptides in the PM of Arabidopsis cell culture were examined using SILAC and titanium dioxide (TiO_2) enrichment along with LC-MS/MS. Kinase activity was found to be the highest at 5–10 min after flg22 or xylanase treatment and decreased after 30 min. At 10 min after treatment, the researchers identified 472 phosphorylated proteins, and a total of 76 of the proteins with 98 peptides were differentially phosphorylated. The phosphorylated proteins include CDPKs, RLKs, respiratory burst oxidase homolog D (RBOHD), and vesicle trafficking associated SYP121 [66]. These phosphorylated residues and their corresponding proteins provided previously unknown information into the membrane phosphorylation events occurring after PAMP treatment. In a separate study, Nuhse and co-workers utilized iTRAQ along with immobilized metal ion affinity chromatography (IMAC) enrichment to identify phosphoproteins involved in flg22 response in Arabidopsis [67]. Identified were 11 peptides with PTMs and significant fold changes. Known defense response proteins NADPH oxidase, RBOHD, and an adenosine triphosphate (ATP) binding cassette transporter, pleiotropic drug resistance8, and penetration3 were shown to be phosphorylated. Further characterization of the phosphorylation sites of RBOHD, S343, and S347 revealed that phosphorylation of the residues is necessary for RBOHD mediated ROS production. Additionally, proteins annotated as being involved in membrane trafficking and ubiquitination such as dynamin, RING-H2 protein, and Arabidopsis toxicos en levadura 6 (ATL6) were also identified as being phosphorylated. These findings help to elucidate regulatory mechanisms of plant innate immune responses via PTMs.

Jones and co-workers analyzed changes in the phosphoproteome of Arabidopsis during *Pst* infection. Phosphoprotein enrichment followed by iTRAQ tagging was employed and proteins that were differentially phosphorylated in soluble *A. thaliana* leaf extracts were identified. Examining proteins with changes during PAMPs response, dehydrin, a protein involved in water stress, and a putative p23 co-chaperone were identified as having a decrease in phosphorylation. Additionally, a plastid-lipid associated protein associated with transportation, a heat shock protein, and a proton-dependent oligopeptide transporter were observed to have an increase in phosphorylation [43]. In addition, a recent study comparing *Lotus*

japonicas roots treated with a nodulation factor and a pathogen elicitor flg22 revealed differential phosphorylation of shared and unique proteins during symbiotic and defense responses [68].

As previously stated, ROS and NO are involved in the signaling mechanisms that characterize PTI, HR and PCD. Their actions are usually carried out through the PTMs of proteins via cysteine residues that act as versatile sulfur switches. The role of redox-based PTMs in plant-pathogen interaction is currently an area of interest. Like other PTMs, the formation of molecular disulfide bonds often affects protein properties. Preliminary redox proteomics was performed to examine pathogen responsive proteins in Arabidopsis [41]. The researchers observed that GST (F6, F7 and F8) had a shift in protein PI, possibly due to redox modifications. Another study with Arabidopsis suspension cells treated with 5 mM H_2O_2 revealed redox induced modifications using two thiol reactive tags, 5-(iodoacetamido) fluorescein (IAF) a fluorescent probe used alongside 2D-DIGE as well as N-(biotinoyl)-N′-(iodoacetyl)-ethylenediamine (BIAM) [69]. A total of 84 potentially redox responsive proteins were identified and they were involved in processes such as metabolism, antioxidation, translation, and protein folding. Parker and co-workers [70] have shown that labeling using the isobaric tags can provide multiplex high throughput analysis of redox responsive cysteines and proteins. When mock treated and *Pst* treated tomato samples were compared, a change in the reporter ion spectra can be compared between labeled samples showing quantitative cysteine PTM changes. This method allowed researchers to quantify redox changes and map the modified cysteines across six samples. The recently developed cysTMT tags have been used to examine potentially redox-regulated proteins [71]. Here cysTMT labeling helped to identify similarities and differences of protein redox modifications in tomato resistant (*PtoR*) and susceptible (*prf3*) genotypes in response to *Pst* infection. A total of 4348 proteins were identified by LC-MS/MS, 90 of which were identified to be potentially redox-regulated. The 90 potential redox-regulated proteins fell into diverse categories including carbohydrate and energy metabolism, biosynthesis of cysteine, sucrose and brassinosteroid, cell wall biogenesis, polysaccharide/starch biosynthesis, cuticle development, lipid metabolism, proteolysis, tricarboxylic acid cycle, protein targeting to vacuole, and oxidation–reduction.

Although several different redox-based PTMs are known to occur in plants (Fig. 9.3), protein nitrosylation is considered as one of the key mechanism regulating protein function [72]. Nitrosylation is a PTM in which a nitric oxide radical oxidizes a free thiol group as well as tyrosine, thus NO produced during plant-pathogen interactions could exert their signaling action through nitrosylation of specific proteins [73, 74]. To examine potentially S-nitrosylated proteins, Arabidopsis suspension cells were treated with NO donors S-nitrosoglutathione (GSNO) and sodium nitroprusside, and leaves were exposed to NO gas [75]. A total of 63 proteins from the GSNO treatment and 52 proteins from the NO treatment were identified as being S-nitrosylated. The proteins were found to be involved in stress, redox regulation, signaling, cytoskeleton structure, metabolism, and photosynthesis and included proteins such as SOD (copper (Cu) and zinc (Zn)), a glutathione peroxidase, elongation factor 1α-chain, actin 2 and 7, fructose

1,6-bisphosphate aldolase, and Rieske Fe-S protein. This study provided information on potential targets of S-nitrosylation as well as the sites where the modification is likely to occur.

Differential thiol nitrosylation during HR was observed in Arabidopsis after avirulent *Pst* (*avrB*) treatment at 0 hai, 4 hai, and 8 hai [76]. A total of 18 differentially modified proteins were identified. For example, RuBisCO in photosynthesis was identified along with an allene oxide cyclase involved in JA signaling. The other

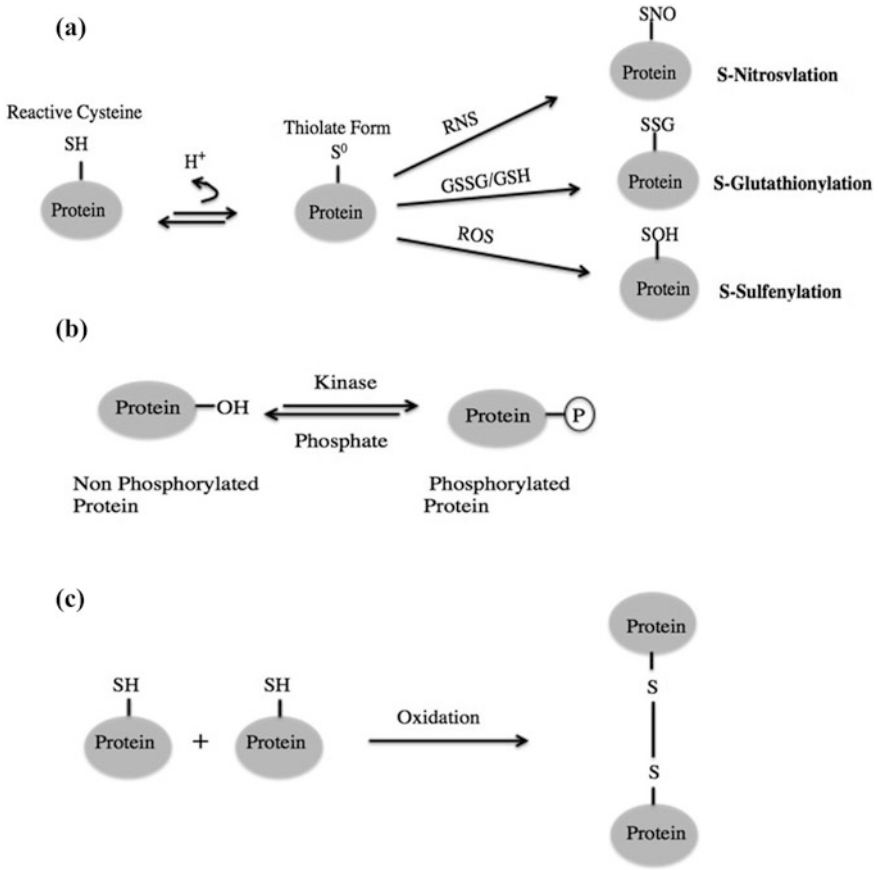

Fig. 9.3 PTM proteomics methods used in plant bacterial pathogen interactions. **a** A reactive cysteine (SH) with a low pka can readily lose a hydrogen ion leading to the formation of a highly reactive thiol S^0, which can react with reactive nitrogen species (RNS) to become S-nitrosylated (SNO), Glutathione disulfide (GSSG)/reduced Glutathione (GSH) to become S-Glutathionylated (SSG) or reactive oxygen species (ROS) to become S-Sulfenylated. These oxidative modifications are reversible. (*Note* Further oxidation of sulfenic acid to sulfinic acid (RSO_2H) and sulfonic acid (RSO_3H) is thought to be generally irreversible.) **b** During protein phosphorylation, phosphate moieties are transferred by protein kinases to serine, threonine or tyrosine residues of proteins. This reaction can be reverse by protein phosphatases that hydrolyze phosphate moieties. **c** Depiction of the formation of a disulfide bond as a result of oxidation of two sulfhydryl groups

processes observed included metabolism with the identification of glyceraldehydes-3-phosphate dehydrogenase and redox regulation including a germin-like protein. In addition, the S-nitrosylated sites on the proteins were mapped. A change in the nitroproteome of Arabidopsis through tyrosine nitrosylation is also evident during HR [77]. With peroxynitrate ($ONOO^-$) treatment, eight proteins were identified as being nitrosylated including the 33 kDa oxygen evolving protein, RuBisCO, and glutamine synthetase 2, proteins involved in photosynthesis, Calvin cycle and glycolysis, and nitrate assimilation. A time course of treatment with avirulent *Pst* (*avrB*) revealed that nitrosylation peaked from 4 hai to 8 hai. The results here indicate that tyrosine nitrosylation may play a role in pathogen response.

9.4 Conclusions and Perspectives

The study of plant-pathogen interactions is a broad and important area of plant biology research. PTI and ETI have been examined extensively in Arabidopsis and to a lesser extent in crop plants. Bacterial responses have been characterized and modeled in order to describe not only how infection occurs, but how bacteria respond to plant defense mechanisms. Progress has been made in the analysis of individual genes and proteins as well as transcriptomic changes however, plant-bacteria proteomics has just touched the surface and secretome as well as PTM proteomic changes due to pathogen infection are still in their infancy. The information gathered from global proteomics and PTM proteomics allows researchers to observe processes underlying pathogen infection and plant defense responses. The knowledge will allow for the connection between molecular networks to be made and further characterized in order to gain a more dynamic perspective on pathogen response mechanisms. Over the past years, proteomics research has markedly contributed to the knowledge of the changes in proteins and associated biological functions in response to pathogen infection however further progress is required. For example, PTMs go beyond phosphorylation, S-nitrosylation, and disulfide bond formation, and those areas have yet to be described in the context of plant pathogen interactions. The area of proteomics and the technology used to characterize global protein changes including PTMs are rapidly advancing, and with those advancements, researchers are able to examine regulatory mechanisms beyond protein level changes. With the fast advancement in high resolution separation, high sensitivity, and versatile mass spectrometers, whole proteome coverage of plants and bacteria is in sight. Large-scale multiplexing (e.g., Neucode [78]), characterization of low abundance proteins, protein-protein interaction, and PTM crosstalk are new directions of proteomics that will allow construction of molecular networks underlying plant pathogen interactions and achievement of the ultimate goal of determining critical nodes and edges in the networks in order to achieve rational engineering/breeding of crops for enhanced yield, quality and defense.

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