

Proteomics of effector-triggered immunity (ETI) in plants

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Abbreviations: ETI, Effector-triggered immunity; T3SE, Type III secreted effector; NB-LRR, nucleotide-binding and leucine-rich repeat

Effector-triggered immunity (ETI) was originally termed gene-for-gene resistance and dates back to fundamental observations of flax resistance to rust fungi by Harold Henry Flor in the 1940s. Since then, genetic and biochemical approaches have defined our current understanding of how plant “resistance” proteins recognize microbial effectors. More recently, proteomic approaches have expanded our view of the protein landscape during ETI and contributed significant advances to our mechanistic understanding of ETI signaling. Here we provide an overview of proteomic techniques that have been used to study plant ETI including both global and targeted approaches. We discuss the challenges associated with ETI proteomics and highlight specific examples from the literature, which demonstrate how proteomics is advancing the ETI research field.

Introduction

The plant immune system can be triggered by the recognition of two broad classes of microbial molecules. PRR-triggered immunity (PTI) is activated following the recognition of conserved microbe-associated molecular patterns (MAMPs) by cell surface plant pattern recognition receptors (PRRs), while effector-triggered immunity (ETI) is activated by recognition of pathogen effector proteins by intracellular nucleotide binding, leucine-rich repeat (NB-LRR) proteins. Both PTI and ETI result in similar immune responses, although the amplitude of the ETI-induced response is often substantially higher and frequently associated with a localized programmed cell death (PCD) response around the site of infection called the hypersensitive response (HR). The recognition of effectors by NB-LRR proteins can be direct where the NB-LRR protein directly binds the effector to trigger ETI, or indirect where the NB-LRRs interact with the host target of pathogen effectors and monitor them for perturbations.^{1,2} An exception to the effector/NB-LRR paradigm for ETI activation

is the recognition of transcription activator-like (TAL) effectors which can transcriptionally activate expression of non NB-LRR genes to activate ETI, but proteomic analyses have yet to be conducted on these TAL ETI “executors.”³

Genetic approaches have been successful at identifying numerous genes required for ETI and have largely built our current understanding of the ETI network. More recently, proteomic analyses of ETI have emerged as a powerful complement to genetic methods by expanding the repertoire of proteins and PTMs responsible for ETI signaling, identifying protein complexes that contain novel ETI signaling components, and providing an overview of the molecular events responsible for manifesting the cellular responses associated with ETI (Table 1 and 2). Additionally, proteomic approaches have provided important insights into the physiology of the ETI response, such as alterations in photosynthesis, lipid metabolism and redox potential. The co-regulation of antagonistic aspects of these physiological responses reveals that they are tightly regulated during ETI.

The technical progress made in analyzing plant proteomes,⁴ general proteomic strategies, theory and instrumentation have been reviewed elsewhere and we will focus on proteomic approaches used to study ETI induced by type III secreted effectors (T3SEs) of *Pseudomonas syringae*.^{5,6} We use the term “global” to refer to studies aiming to identify and quantify proteins from a total cellular extract, whereas “targeted” approaches identify the components of specific protein complexes. This review highlights the contributions that both approaches have made to our understanding of the plant ETI response (Table 1 and Table 2).

Proteomics of ETI

Despite significant technical advances, plant proteomics remains challenging. Plant cells generally have low cytoplasmic volume relative to cell wall mass, with high protease and phosphatase content.⁷ As a result, careful consideration must be paid to the isolation of proteins from plant tissues to preserve both their integrity and PTMs. Further, the predominance of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), which catalyzes the first major step of carbon fixation and is generally considered the most abundant protein on earth, makes it difficult to concentrate plant protein extracts, and necessitates

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a large dynamic range for protein separation, detection and identification.⁴

An additional challenge in the proteomic study of ETI simply involves the induction of the ETI response. Many signaling events in plants occur downstream of perception of small molecules or peptides (i.e., elicitors) by their cognate receptors. These systems can generally be readily induced under experimental conditions by the application of elicitors such as the bacterial flagellin peptide flg22, which is a potent activator of PTI,⁸⁻¹¹ or the elicitation of phytohormone signaling by the application of exogenous hormones.^{12,13} In contrast, receptors mediating ETI are intracellular and generally detect the activity of proteinaceous effectors translocated from pathogens into the host cytoplasm.¹⁴ Therefore, ETI is difficult to induce experimentally, and requires either the delivery of effectors by pathogens or their transgenic expression.

While pathogen delivery of effectors represents the most biologically and physiologically relevant stimulus, these interactions involve a broad range of host response, not all of which are directly associated with ETI. For example, *P. syringae* strains can inject dozens of T3SE,^{15,16} which can alter host physiology and inhibit immune responses, including ETI. Consequently, it can be difficult to deconvolute ETI signaling from other T3SE functions. Consequently, three experimental treatments are typically compared when identifying proteins regulated by ETI signaling,¹⁷⁻¹⁹ including plants inoculated with: (1) *P. syringae* (with its endogenous complement of T3SEs); (2) *P. syringae* + an exogenous T3SE that triggers ETI; and (3) *P. syringae* lacking a functional type III secretion system that is unable to secrete any T3SEs. Further, since ETI often culminates in PCD, its proteomic study requires careful coordination of inoculation and tissue collection to ensure a consistent stage of ETI in all tissues and limited protein loss from PCD. Despite these complications, bacterial delivery of T3SE is usually easily implemented in crop species²⁰ and cell culture,¹⁹ and therefore represents a viable strategy for translational work in non-model plant systems.

ETI can also be induced by transgenic expression of T3SE *in planta*. Since ETI culminates in PCD, T3SE expression is typically controlled by an inducible promoter. A popular system for transgenic delivery of effectors is the dexamethasone (Dex) inducible GAL4 / VP16 / glucocorticoid receptor domain (GVG) system,²¹ which provides a transcription factor that drives expression of the transgene in a corticosteroid sensitive manner. While the GVG system produces substantially higher levels of the transgenic T3SE than would be expected under natural conditions, ETI induced by these constructs generally recapitulates phenotypes seen with bacterial T3SE delivery^{22,23} and has been successfully used to identify genes required for ETI.²⁴ The GVG system allows simple and synchronized ETI induction by small molecule (Dex) application. Furthermore, because only one T3SE is induced, as opposed to the entire complement carried by a *P. syringae* strain, it simplifies experimental design and downstream analysis.

Global Analysis of Protein Abundance and PTMs during ETI

Pathogen Delivery of Effectors

In *Arabidopsis thaliana* (hereafter *Arabidopsis*), the membrane associated NB-LRR protein RPM1 mediates ETI triggered by the *P. syringae* T3SE AvrRpm1 (Fig. 1a).^{25,26} Jones et al.^{17,18} provided the first insight into proteins differentially regulated during RPM1-mediated ETI. Ultimately, 19 proteins were identified as upregulated in response to *P. syringae* pv tomato DC3000 (PtoDC3000) expressing AvrRpm1 (PtoDC3000(*avrRpm1*)), but not PtoDC3000 or PtoDC3000 lacking a functional TTSS (PtoDC3000(*hrpA*)), and therefore uniquely regulated by RPM1-mediated ETI signaling. In particular, proteins involved in redox regulation, lipid metabolism and photosynthesis were identified.

The glutathione-s-transferases (GST) GSTF8 and GSTF9 were AvrRpm1-upregulated while the NADPH quinone reductase (NQR) and 2 cysteine peroxiredoxin PRxB were downregulated. The concomitant up and downregulation of radical detoxifying enzymes suggests a tight regulation of redox status during ETI. The downregulation of NO turnover proteins such as peroxiredoxin is likely associated with the rapid build-up of NO preceding HR.²⁷ The accumulation of ROS during ETI can directly oxidize fatty acids leading to lipid peroxidation, which may be detoxified by NQRs.^{25,28} The downregulation of NQRs suggests that the accumulation of peroxidized lipids may play a role in ETI signaling.

Proteins involved in photosynthesis also respond to PtoDC3000(*avrRpm1*) providing a link between ETI and altered photosynthetic function. Two members of the oxygen evolving complex (OEC) of Photosystem II (PSII), OEC33 K protein and OEC23 K protein, accumulated in response to AvrRpm1 triggered ETI. Additionally, AvrRpm1 also induces the cytosolic accumulation of ferredoxin reductase which normally catalyzes the terminal photosynthetic electron transfer to NADP(+).¹⁸ Together, these suggest ETI might alter photosynthetic electron transport around PSII. Consistent with altered regulation of photosynthesis during ETI, PtoDC3000(*avrRpm1*) causes a reduction in carbon fixation,²⁹ altered photosynthetic electron transport,^{30,31} and over-activation of chlorophyll associated with ROS production and lipid peroxidation.³¹

In another study, Kaffarnik et al.¹⁹ characterized the secretome of *Arabidopsis* suspension cells in response to incubation with PtoDC3000, PtoDC3000(*AvrRpm1*), PtoDC3000(*hrpA*), identifying 13 proteins specifically upregulated by RPM1 mediated ETI. Surprisingly, only three ETI induced secreted proteins have predicted signal peptides. Non-canonical secretion during ETI could result from loss of cell integrity, autophagy and/or membrane trafficking processes that contribute to ETI.³²⁻³⁴

In tomato, the *P. syringae* T3SEs AvrPto and HopAB2 (formerly AvrPtoB) carried by PtoDC3000 induce ETI mediated by the NB-LRR protein Prf and the host kinase Pto (Fig. 1C).³⁵⁻³⁷ The tomato cultivar Rio Grande (RG) consists of two homogenic

Table 1. Summary of Global Proteomic Analyses of ETI-Regulated Proteins

T3SE	NB-LRR-protein	Cellular Fraction/PTM enrichment	Proteomics Approach	Protein IDs ^a	Ref.	
AvrRpm1	RPM1	Chloroplast,Mitochondria,Cytosol	2D-PAGE	GSTF8 (At2g47730) GST79 (At2g30860) OEC33 (At5g66570) OEC23 (At1g06680)	17,18	
AvrRpm1	RPM1	Secreted Proteins	SDS-PAGE iTRAQ LC-MS/MS	Ubiquitin GAPDH(At1g13440/At3g04120) Phosphoglucomutase (At1g23190) Enolase (At2g36530)	19	
AvrPto/HopAB2	Prf	All soluble proteins	iTRAQ 2D-LC-MS/MS	14-3-3 protein (TC217464_3, TC217870_1, TC233482_2) Acyl-binding carrier protein (TC238115_2) Acyl-carrier protein transacylase (TC219533_3)	20	
GVG:AvrRpm1	RPM1	Microsome, Cytosol	2D-PAGE MALDI-TOF	AtRem1.2 (At3g61260) PP2C PIA1 (At2g20630) C2-domain containing protein (At4g34150)	44,45	
GVG:AvrRpt2	RPS2	Plasma Membrane	SDS-PAGE LC-MS/MS Label free quantitation	SOBIR1 (AT2G31880) RIPK (AT2G05940) PIA1 (At2g20630) SYP122 (AT3G52400) SOBER1 (At4g22300) PLD γ 1 (AT4G11850) DGK5 (AT2G20900)	PEPR1 (AT1G73080) WAK1 (AT1G21250) BIK1 (AT2G39660) SNAP33 (AT5G61210) PEN1/SYP121 (AT3G11820) PLD α 1 (AT3G15730)	49
AvrRpm1	RPM1	Phosphorylated Proteins	iTRAQ LC-MS/MS	Rubsico large subunit (AtCg00490)	60	
AvrB	RPM1	S-nitrosylated cysteine	Biotin-Switch SDS-PAGE MALDI-TOF/TOF	Rubsico large subunit (AtCg00490) GAPDH (At3g26650) Triosephosphate isomerase (At3g55440) PsbQ (At4g21280)	65	
AvrB	RPM1	Tyrosine nitration	Immunoaffinity enrichment 2D-PAGE LC-MS/MS	Rubsico large subunit (AtCg00490) PsbO2 (At3g50820) PsbO1 (At5g66570) Fructose-bisphosphate aldolase 1 (At2g39730) Fructose-bisphosphate aldolase 2 (At4g38970)	66	

Note: ^aTomato gene indices as annotated by DFCI – LeGI

genotypes: RG-PtoR, which expresses Pto and recognizes PtoDC3000, and RG-*prf3*, which lacks Prf and is susceptible to PtoDC3000. Parker et al.²⁰ performed a proteomic analysis of Prf mediated ETI by inoculating the tomato cultivars RG-PtoR and RG-*prf3* with PtoDC3000. Ultimately, 550 proteins were identified as uniquely regulated by Prf mediated ETI signaling. Many proteins regulated by Prf were consistent with similar experiments in *Arabidopsis* challenged with PtoDC3000 (*AvrRpm1*), including proteins involved in photosynthesis, redox regulation and lipid metabolism.^{18,20} Similarities in the proteomes induced by AvrRpm1/RPM1 in *Arabidopsis* and AvrPto/HopAB/Prf in tomato strongly imply conserved early signaling events and proteomic changes during the ETI response.

This study also identified three 14-3-3 proteins that were upregulated by Prf mediated ETI.²⁰ 14-3-3 proteins mediate protein-protein interactions in a phosphorylation dependent manner, strongly implying that early ETI signaling events are regulated by phosphorylation and protein-protein interactions mediated by 14-3-3 proteins. Further, 14-3-3 proteins are known to mediate Prf signaling.³⁸ Interestingly, 14-3-3 proteins are emerging

as targets of T3SE that inhibit both PTI^{39,40} and ETI suggesting a central role for 14-3-3 proteins in mediating plant immune signaling.⁴¹

Both studies in *Arabidopsis*¹⁸ and tomato²⁰ were designed to complement previous transcriptome studies.^{42,43} Interestingly, both studies revealed that there was little correlation between protein and transcript abundance in early ETI signaling (4 h post inoculation). However, correlations were observed at later time points (24 h post inoculation) with increases in transcript and protein abundance for Pathogenesis-Related protein 1 (PR1), peroxidases and lipoxygenases.²⁰ It is perhaps not surprising that early ETI signaling events are likely mediated by proteome changes including PTMs such as phosphorylation or S-nitrosylation, protein-protein interactions or by signaling events such as lipid peroxidation, redox, and ROS/reactive nitrogen species generation that precede transcriptional re-programming.

Transgenic Expression of Effectors

Proteins regulated by RPM1-mediated ETI were also investigated using the GVG system to express AvrRpm1.^{44,45} Total protein and proteins from microsomal preparations were used for

Table 2. Summary of Targeted Proteomic Analyses of ETI Protein Complexes

Target	Tag	System	Purification Strategy ^a	Proteins Identified		Ref.
HopN1 (T3SE)	6xHIS	<i>N. benthamiana</i>	In vitro pull-down, Ni ²⁺ -IMAC	PsbQ		72
RPS2 (NB-LRR-protein)	HA-PreScission-Biotin (HBP)	<i>A. thaliana</i>	Biotin/Streptavidin AP	RIN4 (At3g25070) AtHIR1 (At1g69840) AtHIR2 (At3g01290)	BSK1 (At4g35230) BSK8 (At5g41260)	73
Prf (NB-LRR-protein)	SBP-FLAG	Tomato	TAP, Streptavidin AP/ FLAG IAP	Pto Fen	Pth2 Pth3	80
Pto (Monitored by Prf)	FLAG	Tomato	FLAG IAP	Prf Fen	Pth3 Pth 5	
RIN4 (Monitored by RPM1/ RPS2)		<i>A. thaliana</i>	Native IAP	RIPK (AT2G05940)		50
MOS4 (mediates NB-LRR protein signaling)	HA	<i>A. thaliana</i>	HA IAP	AtCDC5 (At1g09770) PRL1 (At4g15900)	MAC3A (At1g04510) MAC3B (At2G33340)	86

Note: ^aAP: affinity purification, IAP: immunoaffinity purification

proteomic analysis. Despite limited protein identification, the accumulation of OEC complex members, GSTs and PRXs, were observed, demonstrating that proteomic changes resulting from transgenic T3SE expression resemble those produced by bacterially delivered T3SE.^{17,18} Similar to previous studies, protein discovery was confounded by high concentrations of rubisco.^{18,44} In order to improve protein discovery, varying concentrations of polyethylene glycol (PEG) were used to selectively precipitate Rubisco from total protein and microsomal preparations. This approach yielded a greater proportion of putative signaling proteins including the remorin AtREM1.2, a C2 domain containing protein and the protein phosphatase 2C (PP2C) induced by AvrRpm1 (PIA1).

Follow-up studies of PIA1 have demonstrated that *pial* plants show increased resistance to PtoDC3000(*avrRpm1*).⁴⁴ The RPM1 interacting protein 4 (RIN4) is phosphorylated in response to AvrRpm1 and is required for AvrRpm1 recognition by RPM1 (Fig. 1A).⁴⁶ However, *pial* plants do not display altered RIN4 phosphorylation during RPM1 mediated ETI implying that the phosphorylation status of an unknown protein participates in regulating RPM1 mediated ETI. The TTSE AvrB also triggers ETI requiring RPM1 and RIN4.²⁴ While PtoDC3000(*avrRpm1*) induces the accumulation of PIA1, PtoDC3000 expressing AvrB does not.⁴⁵ Disrupting *PIA1* also has no effect on PtoDC3000(*avrB*) growth *in planta*, indicating that multiple signaling pathways exist for RPM1-mediated ETI.

The NB-LRR protein RPS2 also interacts with RIN4 and monitors RIN4 for cleavage by the T3SE AvrRpt2 (Fig. 1B).⁴⁷ Both RPS2 and RIN4 are peripheral membrane proteins (Fig. 1).^{46,48} Recently, Elmore et al. characterized the proteomic responses of the plasma membrane during ETI mediated by RPS2 using the GVG system to express AvrRpt2 in *Arabidopsis*.⁴⁹ Ultimately, 235 proteins increasing in abundance and 188 decreasing in abundance were identified during RPS2 mediated ETI. This was the first proteomic study of sufficient depth in *Arabidopsis* to identify the finer details of ETI signaling. For example, protein kinases and phosphatases were overrepresented among proteins upregulated by RPS2 ETI. Kinases promoting ETI (e.g., RPM-1

induced protein kinase,⁵⁰ and suppressor of BIR1) were upregulated alongside PP2Cs, including PIA1, hinting at shared regulation of ETI signaling by RPM1 and RPS2. Interestingly, the pattern recognition receptors PEPR1⁵¹ and WAK1⁵² were upregulated during ETI, as was the PTI co-receptor BIK1,⁵³ highlighting the potential crosstalk between ETI and PTI.

Proteins upregulated by RPS2 during ETI strongly support the role of membrane dynamics during ETI. Proteins involved in membrane trafficking were upregulated in response to RPS2 signaling, including multiple SNARE complex members involved in vesicle fusion.^{32,54} The increased association of normally cytosolic glycolytic enzymes (pyruvate kinase, PEP carboxylase,⁵⁵ GAPDH⁵⁶) with purified plasma membranes would be consistent with autophagy and membrane trafficking turning over cytosolic enzymes during ETI. Phospholipases (PL) upregulated by RPS2 mediated ETI are likely involved in membrane turnover and signaling. Both PLCs and PLDs associated with the release of phosphatidic acid (PA) during ETI were upregulated,⁵⁷ while a PLA₂ known to decrease PA levels and inhibit ETI triggered by the *Xanthomonas campestris* T3SE AvrBsT⁵⁸ was also upregulated. Therefore, antagonistic branches of phospholipase signaling are concomitantly upregulated by ETI. This is similar to upregulation of antagonistic enzymes in protein phosphorylation and ROS signaling pathways and highlights the tight regulation of molecular events during ETI.⁵⁹

ETI Induced Post-Translation Modifications

Given the evidence of altered phosphorylation in response to ETI mediated by RPM1, Jones et al.⁶⁰ examined the quantitative changes in the phosphoproteome of *Arabidopsis* infiltrated with PtoDC3000, PtoDC3000(*avrRpm1*), or PtoDC3000(*hrpA*) by phosphopeptide enrichment. Five proteins were identified as differentially regulated by PTI and one protein by ETI. The large subunit of rubisco showed increased phosphorylation in response to RPM1 mediated ETI, which may contribute to altered photosynthetic activity during ETI.²⁹

A rapid buildup of NO, such as that observed during ETI, can modify cysteine and tyrosine residues by S-nitrosylation or nitration, respectively. Enzyme classes known to mediate ETI

signaling can be regulated by these redox sensitive PTMs, including peroxidoxins⁶¹ and metacaspases.⁶² Further, turnover of S-nitrosylated glutathione compromises ETI⁶³ and is essential for modulation of plant immune responses by salicylic acid.⁶⁴ Given the potential role of differential S-nitrosylation or tyr-nitration during ETI signaling, Romera-Puertas et al.⁶⁵ and Ceconi et al.⁶⁶ investigated the NO redox modified subproteome during AvrB triggered ETI. Most proteins differentially modified by NO redox reactions belong to primary metabolism including components of the PSII OEC, the large subunit of rubisco and three glycolytic enzymes: triose-phosphate isomerase, phosphoglycerate kinase and GAPDH. The carboxylase activity of rubisco and turnover are modulated by S-nitrosylation^{67,68} and S-nitrosylation of GAPDH's catalytic cysteine is known to inhibit enzyme activity, reducing glycolytic flux.⁶⁹ Therefore, accumulation of NO and other ROS might act as a regulator of plant metabolism during ETI.

Targeted Proteomics of ETI Complexes

Proteomics of ETI protein complexes has also been an invaluable tool for the *in planta* study of ETI signaling (Table 2). One common strategy has been the fusion of affinity tags to T3SEs or genetically characterized components of ETI. Affinity chromatography is then used for purification of protein complexes associated with the tagged “bait,” followed by protein identification by mass spectrometry.⁷⁰ This approach can identify proteins that participate in the same biological process as the “bait” and identify immune components that would be recalcitrant to genetic analyses due to redundancy or essentiality.

Proteomics of Effector complexes

The *P. syringae* T3SE HopN1 is a cysteine protease that inhibits cell death in tomato and *Nicotiana tabacum*⁷¹ and ROS production in *Arabidopsis*.⁷² Proteomic investigation of its host targets was performed by *in vitro* pulldown of HopN1. Recombinant HopN1-His₆ was immobilized and incubated with tomato extract, ultimately identifying oxygen evolving complex subunit PsbQ, the water oxidizing complex of PSII, as an interacting protein.⁷² Recombinant HopN1 demonstrated proteolytic activity against PsbQ in thylakoid membranes, while chloroplasts isolated from leaves inoculated with bacteria expressing HopN1 had reduced PSII activity. Silencing of PsbQ in *N. benthamiana* inhibited ROS production and HR during ETI, suggesting that HopN1 inhibits PCD by decreasing PSII activity required for ETI signaling.⁷²

Proteomics of NB-LRR protein complexes

RPS2

In order to identify RPS2 associated proteins, microsomal preparations of transgenic *Arabidopsis* expressing affinity tagged RPS2 were treated with a protein chemical crosslinker prior to affinity purification of RPS2 complexes and protein identification by mass spectrometry.⁷³ Chemical cross-linking of protein complexes allowed for use of harsh detergent conditions to solubilize microsomal pellets, and has been beneficial in purification of transient protein complexes in yeast⁷⁴ and *Arabidopsis*.⁷⁵ While

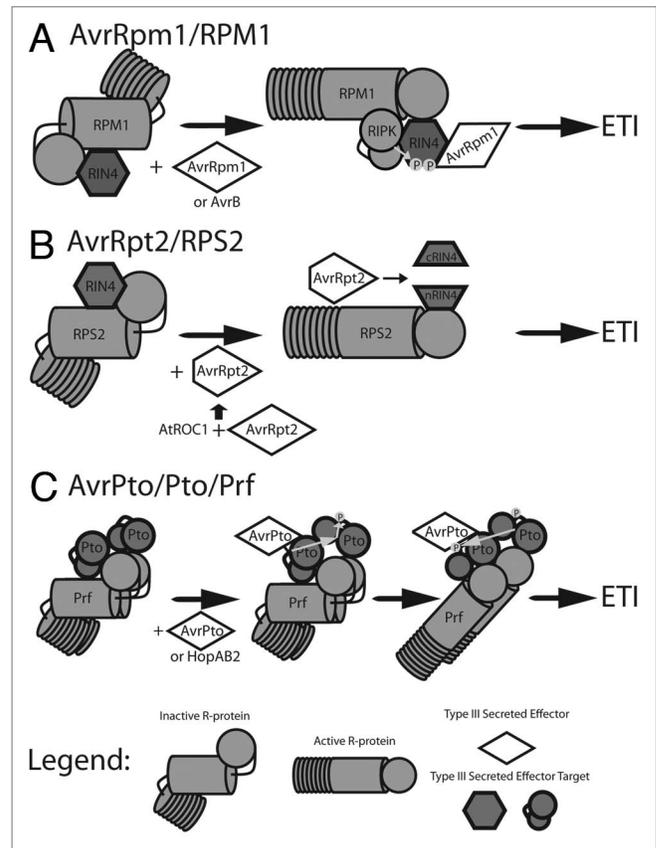


Figure 1. Models of T3SE recognition by NB-LRR proteins to trigger ETI. NB-LRR proteins monitor host proteins targeted by T3SE for modification. Detection of modified host proteins causes NB-LRR conformational change, initiating ETI signaling.¹⁴ (A) The NB-LRR RPM1 monitors the host protein RIN4.^{25,26} Interaction of RIN4 with the T3SE AvrRpm1 or AvrB triggers RIN4 phosphorylation by the host kinase RIPK, activating RPM1.^{50,83} (B) RIN4 is also monitored by the NB-LRR RPS2. The T3SE AvrRpt2 is activated by the host cyclophilin AtROC1, and causes proteolytic cleavage of RIN4 resulting in activation of RPS2.⁴⁸ The RIN4 complexes in A and B are membrane associated. (C) Oligomeric complexes of Pto family host kinases⁸⁰ and the NB-LRR Prf recognize the T3SEs AvrPto^{35,36} and HopAB2.³⁷ Interaction of AvrPto or HopAB2 with a Pto monomer activates Pto kinase activity, causing transphosphorylation and activation of the second Pto monomer. This causes a second transphosphorylation event resulting in phosphorylation of both Pto kinases and activation of Prf.⁸¹

RIN4 was identified as a member of RPS2 complexes, RPM1 was absent. Interestingly, immunoprecipitation of RIN4 in an independent study identified RPS2 but not RPM1, suggesting that either different pools of RIN4 interact with each NB-LRR-protein⁷⁶ or that RPM1/RIN4 interactions are transitory based on RIN4 phosphorylation.⁵⁰

Two members of the hypersensitive induced reaction gene family, AtHIR1 and AtHIR2, were also identified as RPS2 complex members. AtHIR proteins are oligomeric plasma membrane proteins that are associated with detergent resistant plasma membrane microdomains.⁷⁷ These proteins are known to increase in abundance during RPS2 mediated ETI.⁵⁰ Disruption of *AtHIR2* or *AtHIR3* compromises AvrRpt2 induced ETI, while overexpression of AtHIR1, AtHIR2 or AtHIR3 enhances ETI and reduces

PtoDC3000(AvrRpt2) growth. This supports the role of AtHIR proteins in RPS2 signaling and suggests that RPS2 complexes may be organized in plasma membrane microdomains.

The RPS2 complex also includes kinases associated with brassinosteroid (BR) signaling, namely the BR-signaling kinases (BSK) 1 and 8.⁷⁷ These kinases also interact with the PRR FLS2^{77,78} supporting a functional link between ETI and PTI. Further confirmation of ETI/PTI receptor association was also provided by the co-immunoprecipitation of RPM1, RPS2 and RPS5 with FLS2.⁷⁹ Altogether, this demonstrates that receptors from both branches of plant immunity can interact and are likely co-localized in large protein complexes organized into plasma membrane subdomains.

Prf/Pto

A targeted proteomic approach was also used to identify Prf-associated proteins using transgenic tomato expressing affinity tagged Prf.⁸⁰ Multiple Pto kinase family members including Pto, Fen, Pth2, and Pth3 were identified as Prf complex members. Proteomic analysis of affinity tagged Pto also identified Prf, Fen, Pth5 and either Pth2 or Pth3 as members of Pto/Prf complexes.⁸⁰ This implies that Prf forms heterocomplexes containing both Pto and Pth family members, that may allow Prf to recognize T3SEs that potentially target Pto-related kinases.

Characterization of Pto phosphorylation sites during Prf mediated ETI was accomplished through immunoaffinity purification of Pto transiently expressed with AvrPto and Prf in *N. benthamiana*.⁸¹ Double phosphorylation events on Ser198 and Thr199 within the kinase activation domain of Pto were identified as ETI specific. Mutation of either site to residues that do not accept phosphor-transfer had no effect on Prf signaling while a double mutant Pto^{S198A/T199A} inhibits Prf signaling. Nevertheless, expression of a phosphomimetic Pto^{S198D/T199D} does not trigger T3SE independent ETI, indicating that Pto phosphorylation alone is not sufficient for ETI signaling, rather, Prf mediated ETI requires a combination of Pto phosphorylation and T3SE interaction. Consistent with this, the kinase inactive, double phosphomimetic mutant Pto^{D164N/S198D/T199D} can still recognize AvrPto, whereas the kinase inactive Pto^{D164N} does not. Therefore, both Pto phosphorylation and T3SE interaction are required for Prf mediated ETI. Currently, T3SEs are thought to trigger Prf signaling by interacting and perturbing one Pto kinase, resulting in transphosphorylation of the perturbed kinase by the reciprocal Pto kinase of the Prf/Pto oligomeric complex. Critically, such a model provides a mechanism for signaling through Prf heterocomplexes containing Pto kinase family members lacking kinase activity.⁸² Theoretically, inactive family members Pth2–5 may act as a molecular “trap” or decoy for T3SEs in the Prf heterocomplex. Interaction of T3SEs and the inactive Pto family member may induce transphosphorylation by the active family members Pto or Fen to trigger Prf signaling.

Proteomics of NB-LRR associated proteins

RIN4

Targeted proteomics of RIN4 complexes have provided insight into the mechanism underlying ETI signaling. Complexes containing RIN4 were isolated from transgenic *Arabidopsis* expressing DEX inducible AvrRpm1, identifying the RPM1-induced protein

kinase (RIPK) as a RIN4 interacting protein.⁵⁰ Disruption of RIPK compromises ETI triggered by AvrB, and to a lesser extent AvrRpm1.^{50,83} RIPK phosphorylation sites on RIN4 were mapped by mass spectrometry to Thr21, Ser160 and T166. Transient co-expression of RPM1 and phosphomimetic RIN4^{T21D/S160D/T166D} or RIN4^{T166D} induces HR in *N. benthamiana*, while disruption of RIPK in *Arabidopsis* reduces RIN4 phosphorylation in response to AvrRpm1 and AvrB.⁵⁰ These studies show that phosphorylation of RIN4 by RIPK at T166 positively regulates RPM1 mediated ETI.

MOS4

Arabidopsis plants with constitutively active forms of the NB-LRR protein SNC1 display enhanced disease resistance.⁸⁴ In addition, constitutive SNC1 activity also results in smaller stature of *Arabidopsis* plants and constitutive expression of defense-related genes. Signaling components downstream of SNC1 have been identified by forward genetic screens for suppression of small stature and constitutive defense gene expression, termed Modifier of *snc1* (MOS) genes. One SNC1 suppressor, MOS4, interacts with a three proteins by yeast-two-hybrid that form a MOS4-associated complex (MAC).⁸⁵ The MAC protein AtCDC5 is a transcription factor homologous to yeast and human proteins involved in RNA processing and splicing. In order to determine if the MAC complex contains more than the three core members, proteomic analysis of transgenic *Arabidopsis* expressing affinity tagged MOS4 was used to identify MAC interacting proteins.⁸⁶ In addition to the three core proteins, another 22 proteins were identified as MAC members including 19 proteins that showed homology to members of the Nineteen Complex (NTC) in yeast and humans. This complex plays roles in spliceosome assembly, DNA repair and cell-cycle progression. Two MAC complex members, MAC3A and MAC3B, are homologous to the founding member of the NTC in yeast. *Arabidopsis mac3a/mac3b* plants have compromised immunity to PtoDC3000 and PtoDC3000 expressing the ETI inducing T3SEs AvrRps4 or AvrPphB, implying a role for the MAC in both PTI and ETI. Further, *mac3a/mac3b* mutants suppress constitutive SNC1 activation, confirming its role downstream of SNC1 in mediating ETI.⁸⁶ Currently, it is unclear if MAC mediates ETI through altered transcription or splicing. However, the role of MAC in ETI signaling suggests that RNA processing and/or splicing play crucial roles in ETI.

Conclusions

Our current molecular understanding of plant ETI has been shaped by genetic studies. More recently, proteomic approaches have expanded our knowledge of the players and mechanisms involved in ETI using both global and targeted proteomic approaches (Table 1 and Table 2). Despite these advances, the proteomics of ETI still faces important challenges. ETI complexes are often membrane associated and their study incurs the challenges faced by researchers studying membrane proteins, including extraction and maintenance of complex integrity during purification. It is unclear if complexes isolated from plasma membrane microdomains represent protein complexes

maintained by protein-protein interactions or bridged together by common lipid properties maintained during extraction procedures. Although significant advances have been made in global analyses, abundant proteins still confound deep protein discovery in many studies. Consequently, global proteomic analyses have failed to identify components of ETI that have been uncovered using genetic approaches. These limitations are likely due to their low abundance, subcellular localization and/or their lack of changes in abundance during ETI signaling. Rather, global analyses to date have provided insight into the output of ETI including changes in photosynthesis, redox regulation and lipid metabolism rather than signaling mechanisms. On the other hand targeted proteomics approaches, including affinity purification of known immune components, have been more successful at advancing our knowledge of ETI signaling, but lack the breadth of responses revealed by global approaches.

Future advances in plant proteomics will require continued application of the most advanced mass spectrometry technologies and sample preparation techniques. This may involve the development of a repository of contaminant plant proteins identified using affinity purification-mass spectrometry analogous to the

contaminant repository for affinity purification (the CRAPome) developed for humans and yeast.⁸⁷ Despite current limitations, there is no doubt that proteomic approaches form a crucial component for future advances in the study of ETI signaling. These will include the characterization of more NB-LRR complexes, comparative proteomic analyses of ETI induced by different T3SE as well as the temporal and spatial dynamics of the ETI response. The proteomic age of ETI research should continue to increase in momentum as access to proteomic facilities, latest technologies and expertise continues to increase. Currently, the field is perfectly positioned to build on the foundation provided by classical genetics, and to complement advanced genomics approaches provided by next-generation sequencing technologies.

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