

The Plant Host–Pathogen Interface

Cell Wall and Membrane Dynamics of Pathogen-Induced Responses

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ABSTRACT: Perception of pathogens by their hosts is the outcome of a highly coordinated and sophisticated surveillance network, tightly regulated by both host and pathogen elicitors, effectors, and signaling processes. In this article, we focus on two relatively well-studied host–pathogens systems, one involving a bacterial–plant interaction (*Pseudomonas syringae*–*Arabidopsis*) and the other involving an oomycete–plant interaction (*Phytophthora sojae*–soybean). We discuss the status of current research related to events occurring at the host–pathogen interface in these two systems, and how these events influence the organization and activation of resistance responses in the respective hosts. This recent research has revealed that in addition to the previously identified resistance machinery (R-proteins, molecular chaperones, etc.), the dynamics of the cell wall, membrane trafficking, and the actin cytoskeleton are intimately associated with the activation of resistance in plants. Specifically, in *Arabidopsis*, a possible connection between the actin machinery and R-protein-mediated induction of disease resistance is described. In the case of the *P. sojae*–soybean interaction, we describe the fact that a classical basal resistance elicitor, the cell wall glucan elicitor from the pathogen, can directly activate host hypersensitive cell death, which is apparently modulated in a race-specific manner by the presence of R genes in the host.

KEYWORDS: disease resistance; defense; innate immunity; effector; elicitor; hypersensitive cell death; *Arabidopsis thaliana*; Glycine max, soybean; *Pseudomonas syringae*; *Phytophthora sojae*; isoflavone; phytoalexins; cytoskeleton; actin

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INTRODUCTION

Plants have evolved two primary defense systems to combat pathogen attack.¹ One of the plant's first responses to invading microbes is *basal resistance*, also sometimes called general resistance or innate immunity. Basal resistance is triggered by the recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are defense elicitors often associated with the cell surface of pathogens. While often polymeric, active fragments of PAMPs are released upon contact with the host. While the molecular mechanisms underlying basal resistance are currently not well understood, its induction is believed to be associated with MAP kinase signaling, transcriptional induction of pathogen responsive protein and secondary product defense genes, deposition of polymeric wall reinforcements (e.g., callose, lignin, and other phenolic polymers) at sites of infection and, ultimately, abrogation of pathogen growth. In addition to basal defenses induced by PAMPs, plants also defend themselves by *effector-triggered plant resistance*. As the primary tenants of gene-for-gene resistance, these effectors play dual functions as both virulence and avirulence factors.^{2,3} In the absence of the cognate resistance (R) proteins, effectors can function to disable host basal defenses and to release nutrients from host cells, rendering the host susceptible to pathogen proliferation. However, if these effectors are recognized by plant surveillance systems, usually the R gene and/or associated host proteins, they activate defense responses, usually manifested as a form of programmed cell death called the hypersensitive response (HR).

In this article we discuss these two broad forms of resistance and their regulation in two very different, but relatively well-delineated host–pathogen systems. The first is that between the bacterial pathogen *Pseudomonas syringae* and its host, *Arabidopsis thaliana*. The second involves the interactions between the oomycetic pathogen, *Phytophthora sojae* and its host, *Glycine max*. In both cases we present evidence for interesting new findings on the mechanisms underlying these classical forms of resistance. In the case of the former system, a possible connection between the actin machinery and R-protein-mediated induction of disease resistance in *Arabidopsis* is described. In the case of *P. sojae*–soybean interactions, we describe the fact that a classical PAMP, the cell wall glucan elicitor from the pathogen, can directly activate cell death, which is apparently modulated in a race-specific manner by the presence of R genes in the host.

***PSEUDOMONAS SYRINGAE-ARABIDOPSIS THALIANA:* RESISTANCE, EFFECTORS, AND ACTIN**

Gram-negative pathogens of both plants and animals share a number of common features, which has aided not only in the elucidation of their respective activities, but also in determining host targets and mechanisms of resistance

signaling.²⁻⁴ For example, it has recently been demonstrated that *Pseudomonas syringae* effector proteins AvrPphB and AvrRpt2 are members of a class of type III secreted cysteine proteases, functionally related to the YopT effector from *Yersinia pestis*, a bacterial pathogen of humans. YopT is a cysteine protease that cleaves the Rho family GTPase, which causes the disruption of the actin cytoskeleton and contributes to the inhibition of phagocytosis of the pathogen.⁵ As we will discuss below, research in our lab has identified a possible connection between the actin machinery and R-protein-mediated induction of disease resistance in *Arabidopsis*.

Effector-Triggered Plant Resistance: R-Proteins

Numerous R genes have been cloned from a wide range of plant species.⁶ The largest class cloned to date is the family encoding proteins that contain a nucleotide-binding (NB) site and leucine-rich repeat (LRR) domain. Interestingly, the nucleotide-binding motifs in plants share sequence similarities with regions of apoptosis regulators, such as *CED4* from *Caenorhabditis elegans* and *Apaf-1* from humans.⁶ This suggests that R protein function may require, at least in part, the activity associated with ATP binding and/or hydrolysis,⁷ and by analogy, may serve as a signal transducer of cell death-related responses. The carboxy-terminal domain of this class of resistance proteins is represented by the LRR, which is typically 20–30 amino acids in length, and appears to be involved in the formation of protein–protein interactions. The NB-LRR class of R genes can be further divided into coiled-coil (CC)-NB-LRR and toll-interleukin-1 receptor (TIR)-NB-LRR according to their N-terminal domain. Evidence suggests that the N terminus influences the requirement for downstream defense response components. In the model plant system *Arabidopsis thaliana*, over 150 proteins are predicted to be NB-LRR proteins. Collectively, this class of R-proteins determines resistance to bacterial, viral, fungal, and oomycete pathogens. The best-characterized members of the NB-LRR class include members of the CC-NB-LRR subclass: RPS2, RPM1, and RPS5, *Arabidopsis* R-proteins specifying resistance to *P. syringae* carrying the bacterial effectors AvrRpt2, AvrRpm1/AvrB, and AvrPphB, respectively.

Indirect Pathogen Recognition: Surveillance

Although many R genes and their corresponding pathogen effectors have been cloned, the biochemical and genetic relationship(s) between each pair is largely unknown. Previously, plant resistance proteins have been hypothesized to serve as receptors directly interacting with pathogen effectors acting as ligands. However, research over the last 5 years has uncovered a complex surveillance mechanism that coordinates resistance responses in *Arabidopsis*

to a multitude of pathogens.^{1,6,8} Contrary to the ligand–receptor model, it is now evident that bacterial effector recognition and signaling have likely evolved as an indirect mechanism whereby a resistance protein monitors the perturbation of a third plant protein by the enzyme activity of the effector. This process of indirect recognition leads to the activation of plant defense responses.

The best-characterized example of the activation of resistance by way of monitoring bacterial effector activity is that of the *Arabidopsis* protein RIN4. RIN4 is monitored by at least two R-proteins, RPM1 and RPS2. RPM1 and RPS2 have each been shown to physically associate with RIN4 *in planta*.^{9–13} The *Arabidopsis* protein RPM1 recognizes two unrelated *P. syringae* effector proteins, AvrRpm1 and AvrB.^{14–15} When AvrRpm1 or AvrB are delivered to the plant cell, RIN4 is hyperphosphorylated by a yet to be identified kinase. This phosphorylation in turn leads to the activation of RPM1-mediated resistance. Thus, although RPM1 resistance is activated in the presence of either AvrB or AvrRpm1, it is activated through an indirect mechanism (i.e., detection of the modified state of RIN4). It has recently been shown that AvrRpm1 inhibits basal defense responses, presumably through its modification of RIN4 and other host targets.¹ Activation of this signaling mechanism requires the activity of the resistance-associated protein NDR1.

Loci Required for Defense Signaling

Genetic screens to identify suppressors of plant resistance genes have identified several important loci including: *NDR1*, *EDS1*, *PBS1*, *PAD1–4*, *RAR1*, and *Hsp90*.^{1,6} The *NDR1* locus is required for RPS2, RPM1, and RPS5 function (members of the coiled-coil + nucleotide-binding site + leucine-rich repeat class of proteins), while *EDS1* is required for RPS4 function. The *RAR1* gene is required by all four resistance loci, while the *PBS1* gene, which encodes a protein kinase, is only required for *RPS5* function.

Of the aforementioned loci, *EDS1* and *NDR1* are among the most identifiable for their respective (and somewhat divergent) roles in resistance mediated by members of the largest class of resistance proteins, the NB-LRR class. *EDS1* has been extensively characterized for its contribution to the TIR-NB-LRR class of resistance proteins, among which include RPS4 and the loci conferring resistance to *Hyaloperonospora parasitica*, RPP2/4/5/21. Conversely, *NDR1* has been shown to be required for the CC-NB-LRR class of resistance proteins.¹⁶ Among these are RPS2, RPS5, and RPM1. Although *EDS1* and *NDR1* appear to function in divergent resistance pathways mediated by members of the NB-LRR family of resistance proteins, there are likely shared points of convergence; both in the initial perception of pathogens, as well as resistance signaling and cell death. While the role *EDS1* plays in disease resistance has been better characterized, the function of *NDR1* remains enigmatic.

Recent work has demonstrated that NDR1 is a plasma membrane-localized, glycosylphosphatidyl-inisitol (GPI)-anchored protein whose expression is required for resistance to *P. syringae* DC3000 expressing the bacterial effector proteins AvrRpt2, AvrB/AvrRpm1, and AvrPphB. However, the detailed biochemical mechanism whereby NDR1 transduces defense signaling is largely unknown.

Actin Dynamics and Resistance

A primary focus of research in our laboratory is the understanding of host processes both required for pathogen recognition, as well as those processes that are directly targeted by the pathogens themselves. Research in this area has led to the identification of dozens of host processes that are presumably targeted by pathogens during infection. Preliminary data in our lab suggest that one such process targeted by plant pathogens is the actin cytoskeleton.

Given the central role the actin machinery plays in the innate immune response in mammals, we hypothesized that the actin cytoskeleton in plants likely plays a central role in host defense responses, and too, may in fact represent a virulence target, much as is the case in mammals. As a general process, the actin cytoskeleton of plants has been demonstrated to play a role in a variety of processes, among which include membrane trafficking, flowering, development, and disease resistance. Taking the latter into consideration, the role of actin depolymerization in mammals in response to biotic stress has been well documented, and moreover, the specific targeting of the actin machinery by pathogenic bacteria is a well-characterized mechanism of pathogenicity.

Following the gene-for-gene model for defense activation, resistance in the host plant following perception of the *P. syringae* follows one of two courses. First, in the absence of recognition, the pathogen multiplies freely, leading to increased pathogen growth, disease, and ultimately death of the host plant. Conversely, when pathogen perception occurs, generally the result of a triggering of monitored host defenses (see above), then resistance is initiated, and pathogen growth is abrogated. To determine if the actin machinery is one such host process that has evolved as a pathogen virulence target, and too, may in fact be a general process that the host monitors for perturbations, we asked the question: Would a mutation resulting in the loss of critical machinery of the host actin cytoskeleton result in the plant's ability to detect pathogen infection? Moreover, does *P. syringae* express (and deliver via the T3SS) an effector candidate that does in fact target the actin machinery?

Using a forward genetics approach, we have identified the actin depolymerization machinery as a key regulator in the initiation of defense responses in *Arabidopsis*. Mutational analyses of several members of the actin depolymerization factor (i.e., ADF) family of proteins revealed a breakdown in the activation of defense responses following infection with the bacterial pathogen

P. syringae. Interestingly, the susceptibility phenotype associated with the ADF mutation(s) correlates with the activity of a specific bacterial effector protein: AvrPphB. As noted above, AvrPphB is related to the YopT family of effector proteins, a class of cysteine proteases, which specifically targets the actin machinery in mammalian cells, effectively shutting down the phagocytotic process. In the case of AvrPphB, while the exact mechanism is unknown, activity of the cysteine protease appears to target (either directly or indirectly) the actin cytoskeleton, effectively shutting down the cellular processes associated with intracellular trafficking. In short, our data suggest that in the case of *P. syringae*-*Arabidopsis*, depolymerization of the actin cytoskeleton is likely required for resistance.

A BASAL RESISTANCE ELICITOR FROM *PHYTOPHTHORA SOJAE* ACTIVATES HR CELL DEATH IN SOYBEAN

Early Foundations of the Soybean–*P. sojae* Interaction

The *P. sojae*–soybean association is one of the earliest host–pathogen systems closely examined at a physiological, cellular, and biochemical level. Very early work led to a thorough characterization of the secondary product pathways leading to the soybean phytoalexins,¹⁷ the pathogen elicitors of these pathways¹⁸ and the physiological events regulating responses to infection and elicitor treatment.^{19,20} The cell wall glucan elicitor (WGE) from *P. sojae* was also one of the first PAMPs identified. Originally identified as an elicitor of the soybean phytoalexins, the glyceollins,¹⁸ as we will see here it is a remarkably global defense elicitor.

The *P. sojae*–soybean association is also a very rich one genetically, with a series of Rps resistance genes providing race-specific resistance to many defined races of the pathogen. Although WGE was found to induce the soybean phytoalexins, the glyceollins, in a race-cultivar nonspecific manner,¹⁸ making it a typical PAMP or elicitor of basal resistance responses, the glyceollins were nevertheless induced in a race-specific manner in infected tissues. Thus, the *P. sojae*–soybean interaction was from the very beginning an intriguing system in which to study the connections between race-specific and basal resistance.

Recent Advances in the Cellular Biochemistry of Soybean –*P. sojae* Interactions

Due to their simple cellular architecture and ease of manipulation, cotyledons have been an organ of choice for cellular, biochemical, and molecular work on soybean–*P. sojae* interactions. Their use was instrumental in defining the multiplicity of responses to infection and WGE treatment and their regulation (for reviews see Refs. 21, 22). Work with cotyledon tissues demonstrated

that WGE-induced defense responses in soybeans are very global and include multiple secondary product (phenylpropanoid/isoflavone) and pathogenesis-related (PR) protein defense responses that are orchestrated in a sophisticated manner in different cell populations proximal and distal to the point of inoculation or elicitor treatment.^{21–23} It was clear from these studies that the WGE from *P. sojae* was a central player in these various defense responses and a large number of studies focused on this elicitor. Secondary product responses to WGE include the formation of conjugates of the isoflavones daidzein and genistein.²⁴ Genistein is directly toxic to *P. sojae*,²⁵ and daidzein is a precursor for the phytoalexin, glyceollin, which also subsequently accumulates in WGE-treated tissues. Phenolic polymers (lignin and suberin) derived rapidly from early phenylpropanoid precursors can also accumulate to massive levels in elicitor-treated cells.²⁶ Thus, the secondary product responses include the formation of two antibiotics and the reinforcement of a potential cell wall barrier. WGE also leads to the activation of expression of genes for various PR proteins,²³ including PR-1a, PR-2, PR-4, PR-6, and PR-10.

In addition to the very global effects of WGE on the activation of defense responses, a possible connection of its activity to hypersensitive cell death was suggested in studies on a phenomenon called elicitation competency.²⁷ It was discovered that the activation of accumulation of the glyceollins by WGE required proximity of treated cells to either wounded or HR dying cells. In the absence of wounding or HR dying cells, WGE induced the accumulation of the isoflavone daidzein, the precursor of glyceollin. It was hypothesized that entry into the cell death program was required for the activation of elicitation competence. Other connections of WGE activity to race-specific resistance and HR cell death were suggested from genetic studies in which it was discovered that elicitation competence was strongly conditioned by the presence of several Rps resistance genes.²⁸

Gene Silencing Reveals Unexpected Connections among Isoflavones, WGE, and the Activation of Race-Specific Hypersensitive Cell Death

In the past few years, we have employed *Agrobacterium rhizogenes*-based RNAi gene silencing^{29,30} to extend the various findings in the model cotyledon system to roots, the primary and economically most important target for *P. sojae* infection. Gene silencing has been highly effective in allowing us to determine the importance of various candidate genes in both race-specific and basal resistance in roots and has confirmed all of the major aspects of defense deployment and regulation first described in cotyledons. However, it has also uncovered some unexpected connections between PAMP-induced basal resistance and race-specific resistance pathways.

Recently, we described that the silencing of isoflavone synthase causes a 95–98% reduction in root isoflavones²⁹ and enhanced susceptibility to *P. sojae*,

including an apparent breakdown of race-specific resistance in silenced roots. While it has been established for many years that the isoflavone defenses are earlier and more strongly expressed in incompatible infected soybean tissues,³¹ these results indicated that they may also participate in the *establishment* of race-specific resistance. We thus explored this phenomenon further. We first demonstrated that silencing of chalcone reductase, which led to an equally effective (ca. 95%) decrease in daidzein (but not genistein) pools in roots, also led to a complete breakdown in race-specific resistance,³⁰ suggesting that *daidzein is the critical isoflavone for expression of race-specific resistance*. To determine if the breakdown of race-specific resistance was accompanied by a loss of HR cell death, we examined the effects of chalcone reductase and isoflavone synthase silencing on cell death as measured by Evan's Blue vital staining or yellow autofluorescence and histochemical staining for hydrogen peroxide/peroxidase induction. All three of these often used protocols for following HR cell death demonstrated that isoflavone synthase or chalcone reductase silencing both led to complete suppression of cell death and the associated hydrogen peroxide/peroxidase activation in lines carrying resistance genes at the Rps 1 locus.³⁰

Together, these results suggested a very tight association of cell death and race-specific resistance to isoflavone accumulation in these Rps lines. Moreover, they demonstrated a connection between isoflavone accumulation, AOS production, and/or increased peroxidase activity. The connection of isoflavones to cell death is also consistent with the fact that lactofen, which induces massive accumulations of the isoflavones³² also upregulates isoflavone synthase and chalcone reductase mRNA prior to the induction of a form of programmed cell death in soybean.³³ The implications of these observations were very important in that they suggest that the *isoflavones are playing far more than a simple antibiotic and lesion-limiting defense role and in fact appear to play a more complex role in hypersensitive cell death and associated reactions*.

WGE Directly Induces Isoflavone-Mediated Cell Death in Soybean Roots and Silencing of Release of Elicitor Fragments from WGE Blocks the HR and WGE-Induced Cell Death

WGE is the major pathogen elicitor of daidzein and glyceollin.²⁴ We thus reasoned that it might participate in some way in isoflavone-mediated cell death. Roots of all soybean lines so far examined, regardless of the presence of an Rps resistance gene, showed a cell death response to WGE that was characterized by yellow autofluorescence (beginning within 24–36 h) and complete collapse of the tissue by 48 h.³⁰ As with yellow autofluorescent cell death in incompatible infections, silencing of CHR or IFS led to a complete suppression of WGE-induced cell death in roots, confirming that the isoflavone daidzein was required.

The PR-2 class of pathogenesis-related proteins encode endoglucanases that putatively release active elicitor fragments from fungal and oomycetic pathogen cell walls.³⁴ An ethylene-induced and elicitor-releasing endoglucanase has been purified and cloned from soybean.³⁵ While this PR-2 is thought to release active elicitor fragments from the intact cell wall glucan of *P. sojae*. Previously, we have examined the expression of this elicitor-releasing PR-2 in soybean in response to wounding and WGE treatment.²³ In cotyledons, it is constitutively expressed at relatively low levels and strongly upregulated by wounding and WGE. RNAi silencing of the expression of this PR-2 in soybean roots led to strong suppression of mRNA for the gene as measured by qRT-PCR. As did silencing of IFS and CHR, silencing this PR-2 also led to a complete breakdown of race-specific resistance and HR cell death in near isogenic lines carrying the Rps1c and Rps1k genes. Silencing also led to the complete suppression of elicitation of glyceollin by WGE in all soybean lines and to cell death responses to WGE. To test whether the PR-2 silenced phenotype could be complemented, we used a preparation of elicitor fragments prereleased from intact WGE by treatment with a cell-free extract of the PR-2 endoglucanase.³⁶ Enzymatically prereleased elicitor preparations elicited a very strong glyceollin response and cell death in PR-2 silenced roots, biochemically complementing the loss of PR-2 expression. These results suggest that PR-2 actually functions *in planta* in elicitor release, and that PR-2-mediated elicitor release is required for the expression of cell death and race-specific resistance in the soybean-*P. sojae* association. Since WGE is required for the *de novo* accumulation of daidzein and glyceollin, this is also highly consistent with the role of these isoflavonoids in the regulation of cell death and race-specific resistance.

Role of WGE in Induction of the Hypersensitive Response in Incompatible Infected Tissues

The activation of cell death by WGE and the effects of silencing PR-2 on the hypersensitive response in infected tissues, strongly suggest a role of WGE in initiation of hypersensitive cell death in infected tissues. However, given the fact that WGE induces cell death even in lines carrying no known R genes, suggests that either undiscovered R genes exist in the so-called universally susceptible cultivar (Williams) used in our studies, or that they function upstream of the R gene interactions with the corresponding Avr gene effectors released by *P. sojae*. The latter possibility is supported by the fact that *P. sojae* produces an additional potential effector protein, which functions as a virulence factor in suppression of the release of active elicitor fragments by the endoglucanase PR-2.³⁷ If this were the case, then in compatible interactions, *P. sojae* may suppress the activation of cell death by inhibiting release of elicitor fragments. In incompatible infections interactions of the R gene and Avr gene products may nullify this suppression of HR cell death.

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