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RECOGNITION AND RESPONSE IN THE PLANT IMMUNE SYSTEM

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■ **Abstract** Molecular communication between plants and potential pathogens determines the ultimate outcome of their interaction. The directed delivery of microbial molecules into and around the host cell, and the subsequent perception of these by the invaded plant tissue (or lack thereof), determines the difference between disease and disease resistance. In theory, any foreign molecule produced by an invading pathogen could act as an elicitor of the broad physiological and transcriptional re-programming indicative of a plant defense response. The diversity of elicitors recognized by plants seems to support this hypothesis. Additionally, these elicitors are often virulence factors from the pathogen recognized by the host. This recognition, though genetically as simple as a ligand-receptor interaction, may require additional host proteins that are the nominal targets of virulence factor action. Transduction of recognition probably requires regulated protein degradation and results in massive changes in cellular homeostasis, including a programmed cell death known as the hypersensitive response that indicates a successful, if perhaps over-zealous, disease resistance response.

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INTRODUCTION

Specific elicitors of host defense responses are encoded by pathogen avirulence (*avr*) genes. These activate plant defense responses during pathogen infection in both laboratory settings and field studies (59). We focus mostly on the genetics of *R*-mediated disease resistance. This term refers to the genetic interaction of pathogen-derived *avr* genes and corresponding resistance (*R*) genes in plants. *R*-mediated resistance was originally demonstrated by H.H. Flor's work on the flax-flax rust pathosystem (46). Since then, *R* genes have been shown to govern plant-pathogen interactions in a variety of host plants, directing responses toward a broad diversity of pathogens including bacteria, fungi, oomycetes, nematodes, and viruses, and even insects (22). The hallmark of *R*-mediated resistance is specificity; most *R* genes recognize one, or in limited cases two, specific pathogen-derived molecules, encoded by *avr* genes. Thus, the easiest mechanistic interpretation of the genetics in these systems is that the R protein is a receptor for a pathogen-encoded Avr protein ligand. However, as detailed below, there is very little evidence supporting this simple model.

Avr proteins can be recognized in the plant extracellular space (apoplast), as during some fungal infections, or they may be injected into the host cell, as is the case with *Pseudomonas syringae* and other bacterial pathogens that use the evolutionarily conserved type III secretion pilus to deliver disease effectors into eukaryotic hosts (30, 79). The maintenance of *avr* genes in pathogen populations is largely due to the fact that they can act as virulence factors (e.g., they are required for full levels of pathogen growth) on susceptible hosts (reviewed in 79). Thus at the population level, both host and pathogen are in constant evolutionary battle to evolve the ability to recognize and to evade recognition and maintain virulence, respectively. Readers interested in the population genetics and evolution of plant-pathogen recognition are referred to a recent review on this subject (50). In contrast to pathogen-delivered Avr proteins, some experimental systems rely on plant responses to purified, or partially purified, pathogen-derived elicitor preparations. Although these elicitors can trigger pathways similar to those initiated by Avr-R signaling, their relevance to the functional outcome of a host-pathogen response is not well understood and is not discussed in this review (55).

R-mediated recognition triggers highly effective resistance, stopping pathogen growth (termed an incompatible interaction, the plant is resistant, the pathogen, avirulent). Absence of specific recognition allows pathogen growth and spread (termed a compatible interaction, the plant is susceptible, the pathogen, virulent). However, even in the absence of specific recognition, the plant defense system is activated to a certain level (basal defense) limiting the extent of disease.

R-mediated recognition in most cases leads to a hyper-activation of basal defense responses, and often is accompanied by a form of programmed host cell death

called the hypersensitive response (HR). Interestingly, defense responses activated by *R* genes are qualitatively very similar to those activated by virulent pathogens during infection (51). This overlap between defenses activated by *R* genes and those activated by virulent pathogens is clearly demonstrated by the existence of single plant mutations that affect both processes. One of the key questions in the field is how does specific recognition of pathogen-encoded Avr products by R proteins lead to a disease-resistant state that uses many of the same outputs as basal resistance?

In this review we focus on recent work addressing how R proteins recognize pathogen Avr proteins, and how R protein activity is regulated during this process. We also address how R proteins communicate to downstream signaling pathways. We consider whether this communication might convert defense pathways that normally are activated ineffectively during infection into a transcriptional output that is highly efficient at halting pathogen ingress. Due to space constraints, we cannot chronicle all known interactions and studies but instead focus largely on informative newer findings, especially in the excellent Arabidopsis genetic model system that has allowed the identification of many defense-related genes (Figure 1) (53).

A DEFINITIVE REPERTOIRE

The advent of genetic model systems and genome sequencing projects has revealed that plants invest a considerable percentage of their genome to the cultivation of *R* gene families. In Arabidopsis, it is estimated that there are ∼125 *R* genes (of the major structural class detailed below), whereas the rice genome encodes ∼600 genes of this class. The actual function of all but a few of these in plant pathogen interactions is unknown (54). This sequence wealth, combined with forward genetic screens, revealed that *R* genes encode a limited set of structural classes (Figure 2) (39).

R PROTEINS: MASTERS OF THEIR OWN DOMAINS

The vast majority of *R* loci encode the so-called Nucleotide Binding Site-Leucine Rich Repeat (NBS-LRR) proteins. Studies of NBS-LRR proteins have begun to reveal potential functions for each discernible domain. NBS-LRR proteins have distinct N-terminal domains: either a putative coiled coil (CC) or a domain sharing homology with the cytoplasmic domain of Drosophila TOLL and mammalian IL-1 receptor (TIR). There appear to be no TIR-NBS-LRR proteins in rice, although there may be TIR-like domains in other genes. Of the other LRR-containing R protein structural classes, the receptor LRR-kinase proteins (see below) have been assigned functions in normal plant development and hormone perception as well as *R* function (e.g., 154, 161). In contrast, the NBS-LRR class has been genetically linked only to disease-resistance function. Thus, we argue that the NBS-LRR protein class represents the core component of the plant

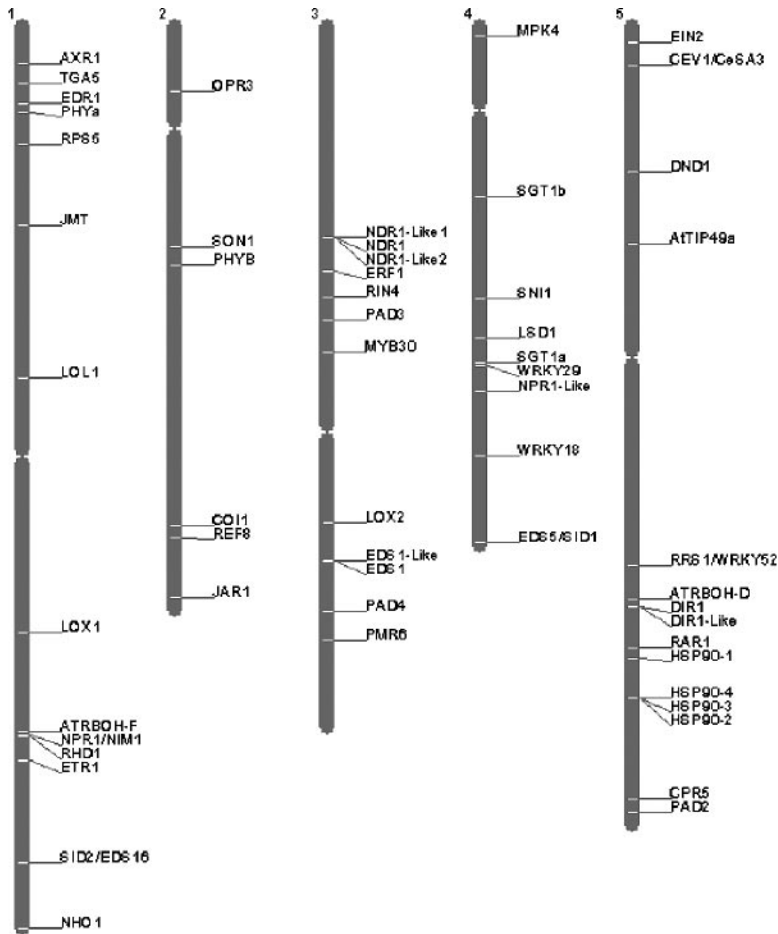

Figure 1 Chromosomal locations of genes implicated in resistance gene defense signaling in Arabidopsis. Each of the five Arabidopsis chromosomes is depicted and the appropriate Arabidopsis gene has been placed on to the physical map using the program Chromosome Map Tool available at http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp. Each gene is represented on the map by its commonly used gene name. Note that not all genes are referred to in the text.

immune system. Other R structures may have been derived from protein families with pleiotropic functions in plant growth and development. It is still possible that all non-NBS-LRR proteins might require the action of an NBS-LRR protein (see below).

The first common feature of the NBS-LRR class is a central nucleotide-binding domain (NBS) that possesses some similarity to the NBS domain of animal pro-apoptotic proteins such as APAF-1 (2, 158). In APAF-1, homo-hexamerization

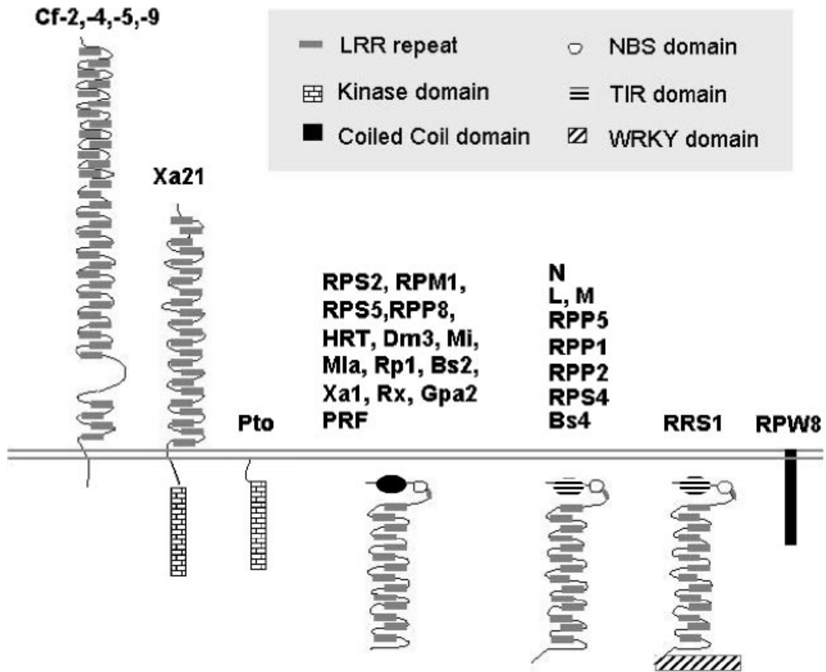

Figure 2 Predicted structures for genetically defined plant resistance proteins. Names above protein structures are examples of R proteins in that respective class. Proteins are shown in relation to the plant plasma membrane. LRR, leucine-rich repeat. Kinase, serine/threonine kinase catalytic core. NBS, nucleotide binding site. TIR, Toll/Interleuken1-receptor-like. WRKY, W-Box DNA binding domain.

leads to ATP binding and hydrolysis as a prerequisite for subsequent signaling. Mutations in the NBS domain generally eliminate *R* function (148). Recent work has demonstrated that both the I2 and Mi NBS-LRR proteins can bind and hydrolyze ATP in vitro (145). Hydrolysis of ATP may therefore play a regulatory role as in APAF-1, although there has been no demonstration of R protein oligomerization to date.

The most common feature of all the R protein classes is the presence of a variable-length LRR domain. LRR domains found in other proteins mediate protein-protein interactions (72). LRR crystal structures from non-R proteins reveal a repeating β strand-α helix subunit structure that folds to form a solvent-exposed face implicated in protein-protein interactions (80). A wealth of DNA sequence data demonstrated that the LRR domains of different R genes are subjected to diversifying selection in predicted solvent exposed residues. In addition, domain-swapping experiments and mutational analysis demonstrated that recognition specificity is governed largely by the LRR domain (reviewed in 39). Taken together, these results suggest that the LRR domain may be responsible for

interacting with Avr proteins directly. Although general demonstrations of direct Avr-R protein interaction have proven elusive, in vitro evidence suggests that AvrPi-ta from *Magnaporthe grisea* can bind to the LRR-like domain in the corresponding rice NBS-LRR R protein Pi-ta (68). In Arabidopsis, the NBS-LRR protein RPM1 recognizes *Pseudomonas syringae* expressing either the AvrRpm1 or AvrB type III effector proteins. A saturation-level screen for loss of RPM1 function led to a large *rpm1* allelic series (95 alleles) (151). Missense mutations in the LRR domain were statistically underrepresented. If the LRR domain mediates binding of Avr proteins, perhaps multiple residues may participate cooperatively in Avr binding, consistent with the observed multi-LRR contacts made by RNAase on the LRRs of RNAase inhibitor (80).

The LRR domain may bind additional host proteins as well. Genetic studies of the *RP2S* gene of Arabidopsis, which recognizes the AvrRpt2 protein from *P. syringae*, demonstrated that allelic diversity in the LRR domain could define a functionally relevant interaction with another host locus (9). This finding is supported by the occurrence of a dominant negative mutation in the LRR domain of Arabidopsis RPS5 (which recognizes the AvrPphB protein from *P. syringae*) that interferes with other *R* functions in Arabidopsis (163).

As mentioned above, NBS-LRRs are generally of one or two varieties; proteins that possess an N-terminal coiled coil domain (CC) or a Toll- IL1-Receptor-like (TIR) domain. It is presumed, based on animal models of these domains, that they are protein-protein interaction domains that may interact with signaling partner proteins. The Arabidopsis and rice genomes possess genes that resemble NBS-LRR *R* genes except that they lack one or more of the R protein domains. These can include TIR-NBS or CC-NBS genes or NBS-LRR genes, among other classes. Several NBS-LRR genes have been demonstrated to undergo alternative splicing, generating variants that encode proteins similar to the truncated *R* genes encoded separately by the genome. The TIR-NBS-LRR N gene in tobacco, which recognizes *Tobacco mosaic virus* (TMV), alters the relative abundance of splice variants during TMV infection. In this study, different splice variants were expressed, and only the WT gene conferred N function (31). In contrast, similar studies using the flax L6 gene demonstrated that alternate splicing, though observed, may not be important for function (6). One alternate hypothesis is that other L6-like *R* gene splice variants can compensate for lack of splicing in L6. Although there is no demonstrated functional significance of *R* gene splice products, the existence of genes that are similar to splice variants makes it tempting to speculate that they have a function in *R* gene regulation.

R PROTEINS DO THE INTRAMOLECULAR TWIST

Loss-of-function alleles have been mapped to all NBS-LRR R protein domains, but some mutations in the LRR domain or NBS domain can lead to constitutively active *R* genes in the absence of pathogen (10, 135). This suggests that R protein function is at least in part under negative regulation. Recent work

suggests that this negative regulation may be in part due to intramolecular protein interactions. Mi is a tomato CC-NBS-LRR protein that governs resistance to both aphids and root-knot nematodes. Domain-swapping experiments between a functional Mi-1.1 allele and a nonfunctional Mi-1.2 allele led to chimeras that triggered constitutive cell death in the absence of pathogen in transient expression assays (64). Interestingly, cell death could be suppressed by co-expression of the N-terminal domain of individual parent genes, suggesting either that intermolecular or *trans* interactions involving the N-terminal domain may regulate R protein activation.

Direct evidence for R protein domain interactions, and support for intramolecular regulation, was provided by immunoprecipitation experiments using domains of Rx, a CC-NBS-LRR that recognizes the PVX coat protein in potato, and the closely related GPA2 protein, a potato NBS-LRR that recognizes nematode pathogens (100). In these experiments, Rx function was restored by co-expression of either the CC-NBS with the LRR domain or the CC with the NBS-LRR domains. These domains co-immunoprecipitated, even though full-length Rx proteins did not, suggesting that this interaction, although assayed in *trans*, was due originally to intramolecular interactions. The association of the CC with the NBS domain required a functional NBS motif, but NBS status did not affect LRR interactions, suggesting that nucleotide binding status regulates domain interactions. Constitutively active NBS alleles of Rx required an LRR domain in order to trigger Avr-independent cell death, suggesting a positive role for the LRR in signaling or associating with signaling components. Taken together, these results suggest that in the absence of pathogen, NBS-LRR proteins may be maintained in an inactive state by intramolecular repression.

R PROTEIN ACTIVATION: GUARDING THE TRIGGERMAN

It is unlikely that R proteins act alone to recognize and transduce Avr-dependent signals, given the lack of data supporting direct interaction between them. Indeed, initial immunoprecipitation studies on the RPM1 and RPS2 proteins of Arabidopsis suggested that several proteins can interact with NBS-LRR proteins (87, 148). One can envision that additional proteins may stabilize R protein complexes, act as cofactors for Avr binding, act as early signaling partners, regulate R protein activity, or combinations thereof. In any case, the identification of proteins that can interact with R proteins has shed some light onto Avr-R protein interactions.

As mentioned above, many *avr* genes also function as virulence factors in susceptible hosts (genotype *r*). With the exception of some virally encoded Avr proteins, in most cases it is not known how an Avr protein acts as a virulence factor or what host genes are required for this function. In a few cases, it is known that Avr proteins can function to inhibit host defense responses in susceptible plants, including R-mediated defense responses (e.g., 121, 155). This suggests that host cellular targets of virulence function could be components of defense signaling

pathways. One suggestion (22, 159) is that R proteins may not recognize pathogen virulence (avirulence) molecules directly, but rather the cellular consequence of their actions in the host cell. This notion has been fleshed out into the "Guard Hypothesis," which at its most basic suggests that targets of pathogen virulence factors are associated with R proteins.

Several lines of indirect evidence support this hypothesis, particularly with respect to bacterial type III effector proteins as virulence factors. First, the site of virulence function action overlaps with the site of R triggering for several pathogen type III effector proteins. For instance, AvrRpm1 and AvrB require localization to the host plasma membrane for virulence functions in susceptible hosts, and similarly, for recognition by plasma membrane–associated RPM1 in resistant hosts (13, 106). Similarly, members of the AvrBs3 family of *Xanthomonas* proteins require nuclear localization for both their virulence and avirulence functions (85, 157, 170). Second, in the few cases where a biochemical function can be ascribed to an Avr protein, biochemical activity correlated with triggering of the R protein, suggesting that the R protein may recognize the products of Avr enzymatic activity and not simply the Avr as a ligand (see below). Third, an Avr protein can affect the activity of partner proteins in the absence of the R protein (see below). The Guard Hypothesis is far from proven. Notably lacking is a demonstrated requirement for host proteins in pathogen virulence. This hypothesis is nevertheless a useful guide and may prove informative in the design or interpretation of experiments.

The potential difficulty in assignment of function to an R partner is illustrated in recent studies of Arabidopsis RIN4, a small protein of unknown biochemical function (93). RIN4, like RPM1 and AvrB and AvrRpm1, is a membrane-associated protein. RIN4 can complex with AvrB and AvrRpm1, leading to RIN4 phosphorylation, even in the absence of RPM1. Loss of wild-type RIN4 blocks RPM1 function, but also prevents RPM1 accumulation, suggesting that RIN4 is required for RPM1 stability. Reduction of RIN4 levels results in plants with constitutive defense responses, reminiscent of ectopic overexpression of NBS-LRR proteins. True null *rin4* plants are inviable. Mutations in RPS2 block *rin4* lethality, proving that *rin4* plants display constitutive defense responses because of ectopic activation of RPS2 (92). Consistent with this, AvrRpt2 triggers degradation of RIN4 in an RPS2-independent manner and overexpression of RIN4 blocks, or slows, initiation of RPS2 function (5, 92). *rps2 rin4* plants still display low-level constitutive defense induction, which is completely suppressed in an *rps2 rin4 rpm1* triple mutant (Y. Belkhadir & J.L. Dangl, unpublished). Thus, RIN4 is also a negative regulator of RPM1 protein function. Both RPS2 and AvrRpt2 can interact in planta with RIN4, demonstrating that either RIN4 regulates activities in two distinct R protein complexes, or that RPM1 and RPS2 are both complexed with the same pool of RIN4. Thus RIN4 negatively regulates the function of at least two NBS-LRR R proteins, is the target of three diverse pathogen effectors in the absence of R protein, and also regulates the relative stability of two different NBS-LRR R proteins, one positively and the other negatively.

At least two NBS-LRR R proteins specifically require serine-threonine kinases for their function. In Arabidopsis, recognition of AvrPphB from *P. syringae* requires both the CC-NBS-LRR RPS5 and the serine-threonine kinase PBS1 (164). Mutational analysis indicates that PBS1 kinase activity appears to be required for RPS5 function (144). PBS1 is not required for any other known NBS-LRR protein function, suggesting specificity. AvrPphB encodes a cysteine protease whose activity is correlated with RPS5 activation (133). Recent work suggests that PBS1 is a target of AvrPphB (R. Innes, personal communication). PBS1 disappears during infection with bacteria expressing AvrPphB in a manner dependent on AvrPphB protease function. This outcome is superficially similar to the activation of RPS2 by AvrRpt2-induced degradation of RIN4, with the exception being that *pbs1* null mutants do not display constitutive RPS5-dependent defenses, whereas *rin4* plants do display constitutive RPS2-dependent defenses. This suggests that if cleavage of PBS1 is important for RPS5 activation, then the conversion of PBS1 to an alternate form rather than a loss of PBS1 per se leads to RPS5 activation. It will be interesting to see how this correlates with any in planta activation of PBS1 kinase activity.

In tomato, the NBS-LRR PRF1 is required for recognition of AvrPto from *P. syringae* (128). AvrPto binds to the serine threonine kinase Pto in the yeast 2-hybrid assay, and Pto is the polymorphic determinant required for AvrPto recognition (131, 146). Constitutively activated Pto kinase mutants can trigger Prf1-dependent cell death in the absence of pathogen, suggesting at least that Pto kinase is inactive in uninfected plants (118). This also suggests that binding of AvrPto may trigger Pto kinase activity; however, a strict in planta activation has not been demonstrated. Pto also can interact with transcription factors implicated in basal defense responses in the 2-hydrid assay, suggesting that Pto may be a link between activation of R proteins and downstream transcriptional activation (58, 177). These intriguing hypotheses await biochemical and genetic testing in planta.

In the tomato-*Cladosporium fulvum* (Cf) system, recognition of extracellular Avr proteins is governed by the Cf class of resistance genes. These encode plasma membrane localized proteins containing an extracellular LRR domain and a short cytoplasmic domain (73, 113). In this system, the tomato *RCR3* gene is required for *Cf-2* recognition of *Avr2*, but not for functioning of related *Cf* genes. RCR3 encodes an extracellular cysteine protease (82). Naturally occurring alleles of *RCR3* were discovered that triggered *Cf-2* dependent auto-necrosis in an allele-dependent manner in the absence of pathogen. Domain swaps with the related Cf-9 protein demonstrated that the LRR domain of Cf-2 was required for RCR3-dependent activation of cell death. Although it is not known if RCR3 and the LRR domain of Cf-2 associate in planta, the genetics suggests that, like RIN4, RCR3 might function both as a true receptor for a pathogen-encoded molecule and as a protein required for R activation. This model might also predict the biochemical activity of the Avr2 molecule.

A recent and exciting example demonstrates how R and Avr protein may in fact interact directly to result in a functional protein complex. The Arabidopsis

RRS1-R protein is an NBS-LRR with the addition of a WRKY transcription factor domain as part of its open reading frame (26, 27). Both it and the "susceptible" RRS-1S allele interact with the *Ralstonia solanacearum* POP2a in a yeast assay. Nuclear accumulation of both RRS1 alleles was regulated by POP2a. Thus, simple physical interaction and consequent subcellular localization cannot be sufficient to trigger resistance. This suggests that functional resistance is only provided by conversion to an active form of RRS1, potentially requiring another host product.

BUILDING A COMPLEX AND TEARING IT DOWN

Recent results suggest that R proteins, in particular NBS-LRR proteins, require cytosolic HSP90 for their function (138). Cytosolic HSP90s are evolutionarily conserved protein cochaperones that regulate the function and assembly of a diverse array of signaling proteins, referred to as client proteins (reviewed in 117). HSP90 molecules can dimerize and can hydrolyze ATP. HSP90 binding to client proteins can either increase or decrease client protein activity (117). Following gene silencing of all HSP90 isoforms, *Nicotiana benthameanum* plants lost all *R* functions tested, including NBS-LRR and other structural classes (D. Baulcombe, personal communication). In a high-throughput genetic screen (151), rare recessive mutations in Arabidopsis HSP90.2 were recovered that block RPM1 function but not that of other CC-NBS-LRR proteins (D. Hubert & J.L. Dangl, unpublished). HSP90.2 plants accumulated markedly reduced levels of RPM1 protein. In wild-type plants, cytosolic HSP90 isoforms could be immunoprecipitated with either RPM1 or RPS2. These findings suggest that R protein complex assembly and possible function can be regulated by HSP90. Null mutations in HSP90.2, however, have no effect on RPM1 function, suggesting that HSP90 function can be complemented by other HSP90 isoforms in the absence of HSP90.2.

Initial studies on RPM1 demonstrated that it disappeared during infection with bacteria delivering AvrRpm1 or AvrB (13). This degradation is probably proteasome dependent, as proteasome inhibitors blocked RPM1 disappearance (D. Mackey, J. Nam & J.L. Dangl, unpublished). A role for the proteasome in NBS-LRR function was recently demonstrated through the loss of *N*-gene function in tobacco plants silenced for components of the COP9 signalsome, a key regulator of proteasome function in multiple organisms (91). If plants do degrade R proteins following activation, it is possible that activation is coupled to R protein degradation to limit the extent of R protein activity, as has been seen for certain transcription factors in animal systems (101).

Cytosolic HSP90 function has been implicated in proteasome-mediated destruction of client proteins (62). For instance, the formation of functional steroid receptors in mammals requires cytosolic HSP90 (117). In the absence of HSP90, steroid receptors cannot bind steroid and are degraded in a proteasome-dependent manner. Following activation, steroid receptors are also degraded by the proteasome.

This strategy might provide the cell with a mechanism for coupling the formation of functional receptor complexes to the degradation machinery by making both processes operate through HSP90. This system would provide a tight mechanism for limiting the effects of spontaneous receptor activation. In the case of R proteins, the lethal nature of misactivation might favor such a control strategy.

How might HSP90 proteins function as molecular links from NBS-LRR protein activation to their down-regulation via the proteasome? HSP90 can be immunoprecipitated with antisera against two proteins implicated in both R signaling and proteasome function, RAR1 and SGT1 (D. Hubert & J.L. Dangl, unpublished). Numerous recent papers highlight the central importance of the *SGT1b* and *RAR1* genes in *R-avr* defense signaling (138). *RAR1* was initially identified in barley, and acts as a nonredundant convergence point for race-specific disease resistance to numerous powdery mildew isolates (136). *RAR1* encodes a small protein with two novel zinc binding domains and a (plant-specific) COOH-terminal extension. The 60aa zinc binding domain (designated CHORD) is found in several animal proteins that have a COOH-terminal domain not found in plant RAR1 proteins—a region of so-called SGT1 homology. The SGT1 protein in yeast is a component of the SCF complex, which is an integral component in protein ubiquitylation.

Arabidopsis SGT1b is required for resistance against at least four separate isolates of *Peronospora parasitica* in Arabidopsis (4, 150). Each of these isolates triggers a distinct *R* gene; both CC-NBS-LRR and TIR-NBS-LRR subclasses are represented. These data strongly suggest that *SGT1b*, like *RAR1* in barley, serves as a convergence point for numerous disease-resistance pathways. Nevertheless, some *R* genes do not require *SGT1b* (4). Arabidopsis has an *SGT1b* homolog, *SGT1a*, that might share overlapping function with *SGT1b* for some defense processes. This will be difficult to address, as *sgt1a sgt1b* double mutants are inviable (K. Shirasu, personal communication). Azevedo et al. (7) demonstrated that *SGT1* is required for resistance to powdery mildew in barley. RAR1 and SGT1b interact in vivo in *Arabidopsis* (7). Furthermore, barley SGT1 interacts in vivo with two *Arabidopsis* E3 ubiquitin ligase subunits, SKP1 and CUL1. SCF complexes have E3 ligase activity, and are required to define substrate specificity for degradation and to deliver substrates into close proximity to proteins with E2 ubiquitylation activity (57). These interactions prompted Azevedo et al. (7) to test SGT1b for interaction with CSN4 and CSN5, two components of the COP9 signalosome. Both of these proteins interacted with SGT1. Care should be taken in interpreting the role of the proteasome pathway mutants strictly in R protein–mediated responses, as some are pleiotropic and affect downstream signaling systems (45).

As mentioned above for *hsp90.2* mutants, RPM1 also does not accumulate in an Arabidopsis *rar1* mutant (151), and RAR1 is required for RPM1 function. RPM1 function is not altered in *sgt1b*, and *sgt1a sgtb* double mutants are lethal (K. Shirasu, personal communication), precluding assessment of RPM1 function in these plants. Although no direct data concerning the degradation of other resistance proteins are available, our data suggest that HSP90 proteins may (*a*) help form

R-containing complexes and hold them in a signaling-competent conformation and (*b*) mediate R protein interactions with components of the proteasome complex.

AFTER THE TRIGGER IS PULLED

There are at least three partially independent pathways leading to the transcriptional reprogramming typically associated with defense activation. Two of these pathways are defined by mutations either in the *EDS1* or *PAD4* (enhanced disease susceptibility) gene or the *NDR1* (non-race specific disease resistance) genes (15, 108). *EDS1* and *PAD4* affect the same spectrum of resistance genes, and EDS1 and PAD4 interact physically in vivo (43). Although both proteins have homology to catalytic lipases, no enzymatic activity has been demonstrated for either protein (43, 178). *NDR1* encodes a probable glycosylphosphatidylinositol (GPI)-anchored protein (B. Staskawicz, personal communication), although nothing is known about its biochemical function. In animal systems, GPI-anchored proteins are found in lipid rafts and are associated with receptor complexes including proteins such as HSP70 and HSP90, and the Sgt1b-like protein p23.

Initially, a simple two-pathway model was proposed based on using *eds1* and *ndr1* mutants to test for loss of specific *R* functions. This work suggested that CC-NBS-LRR-type *R* genes signaled through *NDR1* whereas TIR-NBS-LRR genes signaled through EDS1 (1). Although this model is relatively robust, examples to the contrary have been found. For example, RPP8 and RPP13, two CC-NBS-LRR proteins conferring resistance to specific isolates of *P. parasitica*, both function in the absence of either *EDS1* or *NDR1* (11, 96). Furthermore, double *eds1/ndr1* mutations do not affect RPP13 and only moderately suppress RPP8. This provides evidence that some CC-NBS-LRR proteins can transduce a defense signal through at least a third independent pathway. The *R* genes *RPW8.1* and *RPW8.2*, which confer resistance to numerous powdery mildew isolates, encode coiled-coil–type proteins without NBS and LRR regions (169). These proteins also require *EDS1* for their function, but not *NDR1*. This finding suggests that there may be an NBS-LRR protein in this pathway that ties the RPW8 proteins to the *EDS1*-dependent signaling pathway. Therefore, at least some *R* genes defy simple pathway classification schemes.

It has long been recognized that the hormone-like substance salicylic acid (SA) is required for local and systemic acquired resistance (SAR) (36, 94, 166). SA levels increase in tobacco and Arabidopsis at infection sites during compatible and incompatible interactions (127). Experiments using tobacco and Arabidopsis plants engineered to degrade SA subsequent to infection (NahG plants) provided evidence that SAR as well as local basal resistance and local resistance signaled through some *R* genes are compromised in the absence of SA accumulation (23, 48, 84) (but see below). Similarly, the Arabidopsis *eds5* and *eds16* mutants that are deficient in defense-associated SA accumulation are compromised in some *R*-gene pathways as well as SAR and basal resistance (29, 105, 167). Exogenous application

of SA or SA analogs induces SAR and restores resistance in numerous mutants compromised in signaling steps upstream of SA production (14, 108, 156, 162).

A recent study by Wildermuth et al. (167) suggested that the main route of defense-associated SA production in Arabidopsis involves chloroplast-localized isochorismate synthase 1 (ICS1) encoded by *ICS1/EDS16/SID2*. Its substrate chorismate is provided by the shikimate pathway. Transport of SA from plastids to the cytoplasm may be facilitated by a putatively chloroplast-localized *trans*-membrane protein encoded by *EDS5/SID* (98, 105). An alternative route of SA production may involve the general phenylpropanoid pathway converting shikimate pathway–derived chorismate to the SA precursors Benzoyl-glucose or *o*-Coumaric acid (98 and references therein). However, the significance of *ICS1/EDS16/SID2* for *R* gene–mediated and basal resistance (105, 167) rather suggests a minor role of phenylpropanoid pathway–derived SA for plant defenses (98). Major differences between global gene expression patterns in NahG plants and the *sid2* mutant suggest that NahG has pleiotropic effects beyond elimination of SA (52, 160). Future focus on mutations in *ICS1/EDS16/SID2* and *EDS5/SID1*, which have more defined effects on SA metabolism, will greatly improve analyses of SA-dependent defense signaling events. Mutations in *EDS1* or *PAD4* strongly reduce SA accumulation, suggesting that they act upstream of this important defense signaling molecule. SA also drives increased expression of *EDS1* and *PAD4*, leading to the idea that these proteins act in a positive feedback loop (see also below) (43, 71, 137).

SA perception appears to be modulated by phytochrome signaling (49). In Arabidopsis, *PR* gene activation by SA as well as pathogen-induced HR are dependent on intact phytochrome signaling and light conditions. Growth of incompatible *P. syringae* pv. *tomato* is enhanced in a *phyA-phyB* double mutant. This, together with findings indicating mutual inhibition of SA signaling and jasmonic acid/ethylene signaling (see below) (33), suggests a high degree of crosstalk between regulatory pathways controlling different cellular processes.

Several direct targets of SA have been identified by SA-binding assays (18, 34, 35). A chloroplast-localized carbonic anhydrase (CA) proved to be required for *R*-gene function in tobacco (139). Silencing of *CA* gene expression suppressed *Pto*-mediated HR in tobacco leaves. Along with other SA binding proteins, CA exhibits antioxidant activity and may affect defense signaling by controlling levels of reactive oxygen species (ROI) produced in the oxidative burst.

The oxidative burst is one of the earliest physiological responses during host-pathogen interactions. The triggering of disease-resistance responses in cell cultures results in ROI production within minutes to several hours (66, 88, 112). The primary product of this oxidative burst appears to be superoxide, which dismutates to hydrogen peroxide. These ROI may be directly toxic to invading microorganisms and may contribute to structural reinforcement of the plant cell wall. In addition, a role in defense signaling has been proposed for the superoxide radical (66, 67). It has been recently demonstrated that the majority of ROI generated during the oxidative burst originates from a plant equivalent of the NADPH oxidase complex from mammalian neutrophils (152, 153).

Activation of the oxidative burst is dependent on early changes in fluxes of ions such as calcium (12, 66, 179). In addition, Calmodulin family members and Calmodulin-like domain protein kinases (CDPKs) have been implicated in regulation of the oxidative burst and other defense responses (77, 123, 124). Furthermore, activation of the oxidative burst appears to involve heterotrimeric and small G proteins (75, 129, 142).

Several MAP kinases (MAPKs) are activated within minutes in cell cultures by elicitors and, in particular, by several Avr/R interactions (89, 125). This MAPK activity increase cannot be inhibited by diphenylene iodonium (DPI) and appears therefore to be independent or upstream of ROI production. Building on previously published analyses (see below), a recent dissection of a signaling cascade triggered by the elicitor flg22 identified MEKK1, MKK4a/5a, and MPK3/6 as MAPKKK, MAPKK, and MAPK components of a defense-associated MAPK module in Arabidopsis (3). This module appears to stimulate immediate early flg22 responsive expression of a WRKY-type transcription factor (see below). Transient overexpression of MKK4a, MKK5a or a constitutively active MEKK1 results in enhanced resistance to virulent *P. syringae* and *Botrytis cinerea*. The tobacco orthologs of MKK4/5 and MPK3/6, NtMEK2 and WIPK/SIPK, respectively, appear to relay *avr-R* gene and elicitin-triggered signals (125, 171, 173, 174, 176).

The MAPKs, SIPK, and WIPK at least partially mediate HR cell death (143); (175). Virus-induced gene silencing of SIPK, WIPK, and the MAPKK NtMEK2 attenuates *N* gene-mediated TMV resistance (70). Both SIPK and WIPK are functionally interconnected (70, 90). SIPK appears to be the primary target of NtMEK2, and NtMEK2-mediated activation of SIPK seems to trigger *WIPK* expression. Activity of the newly synthesized WIPK also requires NtMEK2. The tobacco MAPKKK component NPK1 has been shown by virus-induced gene silencing to be required for function of the *R* genes *N*, *Bs2*, and *Rx*, but not *Pto*, *Cf4*, and *Rps2* (69).

Effects on *R* gene–mediated resistance in the *edr1* (enhanced disease resistance 1) mutant, as well as effects on basal resistance in the *mpk4* mutant (47, 65, 111), provide further evidence for roles of MAPK modules in plant defense signaling. In contrast to MEKK1, MKK4a/5a, and MPK3/6 as well as their tobacco homologs, which act as positive regulators of defense responses, EDR1 (a MAPKKK) and MPK4 (a MAPK) appear to be involved in negative regulation. Both the *edr1* and *mpk4* mutants exhibit elevated resistance.

A growing body of evidence implicates nitric oxide (NO) in plant defense signaling. NO is a well-characterized messenger molecule controlling a multitude of physiological processes in animal cells including immune responses (165). NO is required for full *R* gene-triggered HR induction in soybean cells and Arabidopsis (24). Furthermore, NO as well as cyclic GMP (cGMP) and cyclic ADP-ribose (cADPR), which operate downstream of NO in animal cells, activate the defense-associated *PAL* (Phenylalanine ammonium lyase) and *PR1* genes in tobacco (24, 37). Whereas *PAL* and *PR1* activation can be mediated solely by NO (37), induction of HR requires synergistic action of both NO and ROI (24). In animal cells, other downstream targets of NO are cyclic nucleotide-gated channels (CNGCs). In the

Arabidopis *dnd1* mutant, one CNGC family member (CNGC2) is defective. This mutant is compromised in its ability to generate HRs in response to avirulent *P. syringae* (20). A second CNGC family member (CNGC4, HLM1) has been implicated in the control of HR and other defense responses (8). *hlm1* mutants exhibit constitutive basal defense and spontaneous HR development. However, it remains to be demonstrated that plant CNGS operate downstream of NO, as in animal systems.

Recently, in a brute force approach, the plant-inducible Nitric Oxide Synthase (NOS) was cloned (16). This protein, which has all the hallmarks of an animal iNOS, is, in fact, the P protein of glycine decarboxylase (P-GDC). While genetic evidence for a requirement for P-GDC in disease-resistance responses is eagerly awaited, this biochemical breakthrough is important. It is particulary noteworthy that alleles of P-GDC in oats are the targets of a fungal toxin, and that an alternate allele is a resistance gene against a second fungus (103, 104, 174).

A positive feedback loop involving production of ROI, NO, and SA appears to play a central role in the activation of the defense program (24, 25, 37, 137; reviewed in 97, 165). All three messengers appear to act syngergistically in triggering HR and other defense responses. Pretreatment of soybean cells with physiological concentrations of SA potentiates ROI production and HR triggered by avirulent *P. syringae* (137). Similarly, NO potentiates ROI-triggered HR formation in soybean cells (24). Since both ROI and NO stimulate SA biosynthesis and SA potentiates ROI as well as NO-mediated responses, SA appears to act upstream and downstream from ROI and NO. Thus, *R* gene-dependent pathogen recognition appears to trigger a feedback loop amplifying the initial signal and leading to effective activation of downstream defense responses. In addition to ROI, NO, and SA, this feedback loop appears to involve more components that are shared by numerous *R*-gene pathways as well as the basal defense system, such as EDS1, NDR1, and PAD4 (43, 71, 134).

A FLOOD OF GENE ACTIVATION

Transcriptional changes in up to ~20% of the Arabidopsis genome are associated with *R* gene-mediated and basal pathogen resistance. Our knowledge about defense-associated gene expression is being revolutionized by large-scale gene-expression profiling technologies. Strikingly, global gene-expression patterns associated with compatible and incompatible local resistance proved to be quite similar (95, 147). A large number of defense-related genes are upregulated to a comparable extent in both defense situations. *R*-dependent responses, however, are more rapid and specific subsets of defense genes are induced to higher amplitudes. For instance, numerous genes respond in a similar manner to both virulent and avirulent isolates of *P. parasitica* in Arabidopsis. However, some 30 genes were found to be more intensely upregulated during the respective incompatible interaction (95).

Faster and steeper temporal activation of individual *PR* genes during incompatible interactions, as compared to compatible interactions, was historically observed in various systems (61, 140). Simply, *R*-dependent responses appear more efficient than the basal defense pathways operating during compatible interactions. Therefore, subtle differences in the timing and amplitudes of transcriptional activation, rather than profound qualitative differences in global expression patterns, may account for the effective *R*-mediated response. Genes exhibiting faster and more intense upregulation following *R*-dependent signaling may play important roles in disease resistance. Large sequence-indexed populations of Arabidopsis T-DNA mutants (132) will allow systematic testing of such candidate genes for roles in disease resistance. Tao et al. (147) proposed a simple quantitative model with a saturating response curve that approximates the overall behavior of the local defense system. This model (Figure 3) postulates a mechanism common to both *R*-gene and basal defense pathways which converts signal input to gene expression output in a quantitatively determined manner.

GLOBALIZATION OF DEFENSE REGULATION

A key element in controlling SA-mediated gene expression changes is NPR1. For example, expression of essentially all the genes demonstrated by Maleck et al. (95) to be upregulated along with SAR activation (represented by 132 ESTs) is affected in the *npr1* mutant *nim1-1*. Although NPR1 protein is transported into the nucleus in response to SAR induction, and its nuclear localization is required for SAR-induced *PR* gene activation (78), NPR1 itself probably does not act as a transcription factor. However, there is ample evidence for direct physical interactions between NPR1 and members of the TGA subfamily of bZIP transcription factors (TGA-bZIPs) (44, 76). Two functionally relevant TGA-bZIP binding motifs (with a TGACG core motif) are required for *PR1* activation by treatment with the SA-analog 2,6-dichloroisonicotinic acid (INA) (86). One of these motifs, LS7, acts as an activator element, whereas the second one, LS5, appears to function as a weak silencer element. In vitro binding of one TGA-bZIP family member, TGA2, to both LS7 and LS5 was shown to be enhanced in the presence of NPR1 (28). Thus, NPR1 appears to alter the activity of transcription factors rather than directly controlling expression levels of target genes.

In addition to TGA-bZIPs, other transcription factor families seem to be involved in defense gene activation. Members of the large plant-specific family of WRKY transcription factors (41) appear to act upstream and downstream of NPR1 in defense signaling. *NPR1* expression has been demonstrated to be controlled by WRKY factors (172). Mutation of WRKY binding sites in the *NPR1* regulatory region leads to a reduction of basal resistance. A role of WRKY factors downstream of NPR1 in SAR gene activation is strongly supported by the significant enrichment of their recognition site in SAR gene promoters (95). A putative WRKY binding site within the *PR1* promoter (LS4) acts as a strong repressor element (86). At least one member of the WRKY family can act as a transcriptional repressor

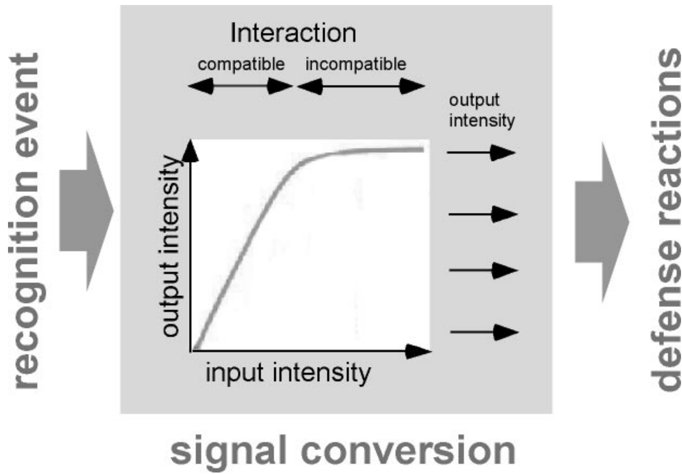

Figure 3 A model to explain the quantitative nature of defense signaling. As proposed by Tao et al. (147), molecular recognition of pathogens by R proteins or (yet unknown) components of the basal defense system generates signal input for a general conversion mechanism. This signal conversion mechanism is common to both *R*-dependent and basal defense pathways and converts signal input to gene-expression output in a quantitatively determined manner. The characteristics of this mechanism are represented by the saturation curve. *R*-independent pathogen recognition generates an input signal of low intensity resulting in a weak output signal. *R* gene-mediated pathogen recognition generates much stronger input signals resulting in high output intensities. The output intensity determines the activity levels of target genes. As a result, *R*-gene pathways activate more rapidly and intensely target gene expression. This regulatory mechanism most likely involves NDR1, EDS1, and PAD4 as well as other signaling components that contribute to both *R* gene-mediated and basal resistance in a quantitative manner, such as additional components of the ROI/SA amplification loop. Modified after Reference 147.

(122). Thus, a general mechanism of NPR1-mediated SAR gene activation may involve derepression by WRKY factors combined with activation by other gene regulators such as TGA-bZIPs or ERFs (ethylene response factors) or additional members of the WRKY family.

Several large-scale expression profiling studies revealed that a multitude of transcription factor genes are expressed in response to a wide variety of different defense–related stimuli (17, 38, 95, 102). Members of the large ERF/AP2-domain, bZIP, homeodomain, Myb, WRKY families as well as other zinc-finger factors were found to be upregulated during multiple incompatible and compatible interactions. Elevated expression of such potential regulator genes in certain defense situations by no means proves a role of the respective factors in these processes. Their upregulation may be an indirect consequence of the activation of the defense

program rather than its cause. However, several independent studies indicated that products of transcription factor genes showing defense-associated upregulation can specifically bind to promoters of *PR*- or other defense-related genes and may participate in their regulation (42, 81, 126, 177).

Transcription factor activity can be linked to upstream signaling events by phosphorylation (74). Both ERF and WRKY transcription factors may be targeted by defense-activated protein-kinases (3, 177). Three ERFs, Pti4, -5, and –6, physically interact with the tomato R protein Pto, a serine/threonine protein-kinase that mediates recognition of strains of *P. syringae*. The flg22-stimulated MAPK module (MEKK1, MKK4a/5a, and MPK3/6; see above) triggers mediate early expression of *WRKY29*. This gene, like other defense-associated WRKY genes (21, 42), appears itself to be regulated by WRKY factors (3), suggesting that MAPK-mediated phosphorylation of early operating WRKY factors may be an important step in flg22-triggered *WRKY29* activation.

In most cases, clear genetic evidence for contributions of defined transcription factors for disease resistance is still lacking. However, recently a novel type of R protein has been identified that may partly act as a transcription factor. The Arabidopsis *RRS1-R* gene, which confers resistance to strains of *Ralstonia solanacearum* expressing POP2a, encodes a TIR-NB-LRR type protein with a C-terminal nuclear localization signal (NLS) and a WRKY DNA-binding domain (27). Certain mutant versions of *RRS1-R* fail to confer resistance to the tested strains of *R. solanacearum*. Whether RRS1-R, in fact, directly regulates gene expression needs to be determined but if this were proven, RRS1-R would constitute an extremely condensed signaling pathway, combining an R-protein type receptor unit with a WRKY-type transcription factor unit in one protein. The fusion of key domains of separate pathway components into a single polypeptide chain in RRS1-R is a possible manifestation of the "Rosetta Stone principle" (83). The chimeric nature of RRS1-R may hint at a general hierarchy of defense pathways, where signals are relayed from R-protein type receptors to WRKY-type (or other) transcription factors, resulting in output of an appropriate gene expression.

Despite the availability of large populations of sequence indexed T-DNA or transposon insertion mutations, reverse genetics approaches have so far not produced much evidence for the involvement of distinct transcription factors in disease resistance. One reason may be that those factor types implicated in defense regulation by circumstantial evidence, such as WRKY, ERF/AP2-domain, bZIP and Myb factors, are encoded by unusually large gene families in Arabidopsis (120). Members of these families may have overlapping functions and disruption of individual candidates may not sufficiently affect gene expression and physiological responses to produce a detectable defense-related phenotype. Combination of single insertion mutations in double, triple, or higher-order mutants may lead to more comprehensive effects and clear phenotypes.

The wealth of gene-expression data generated using GeneChips, microarrays, and other RNA-profiling methods facilitated novel strategies for the discovery of transcription factors of the defense program. Several independent studies demonstrated the identification of highly conserved sequence motifs in promoters

of coregulated genes (17, 63, 95). In addition to motifs already known to function as *cis*-elements, novel motifs have been identified (60). In some cases, function of these novel motifs has been proven (60). Such conserved promoter motifs can be used to clone their potential cognate transcription factors by common screening procedures, such as the yeast one-hybrid system or southwestern screens. Candidate factors can then be tested for their in vivo roles using Arabidopsis insertion mutants. This strategy may have some advantages compared with conventional reverse genetics approaches, as potential molecular and macroscopic phenotypes of the tested insertion mutants are already known.

JASMONIC AND ETHYLENE-DEPENDENT DEFENSE SIGNALING PATHWAYS

Recent studies unmasked SA-independent disease resistance mechanisms in Arabidopsis that are mediated by jasmonic acid (JA) and ethylene (ET) (reviewed in 33; 114, 119). One SA-independent resistance pathway, termed ISR for induced systemic resistance, is triggered by biocontrol bacteria applied to Arabidopsis roots (115). Recent epistasis analysis demonstrated that this resistance is dependent on the ET and JA response pathways (116).

ET and JA regulate expression of genes encoding antimicrobial peptides such as thionin and defensin (40). Thionin gene expression is upregulated by methyl jasmonate and is down-regulated by the ET-insensitive *ein2* and JA-insensitive *jar1* mutations. Similarly, *pdf1-2 (*plant defensin1-2) expression is induced by JA and necrotrophic pathogen infection (109, 110). This induction is eliminated in the JA-insensitive *coi1* mutant and *ein2*. Neither thionin nor *pdf1-2* gene activation is affected by NahG expression, suggesting SA independence. However, epistasis analysis has revealed evidence for antagonism and crosstalk between the SA-dependent and the JA/ET-dependent defense pathways (reviewed in 33). ISR requires NPR1, which also operates downstream from SA (116). Furthermore, constitutive broad-spectrum disease resistance in the *cpr5* and *cpr6* mutants requires components from both SA- and ET/JA-dependent pathways (19). Constitutive resistance in *cpr5* and *cpr6* is partially affected by the *npr1* mutation and completely abolished by the *eds5* mutation that suppresses SA accumulation. Hence, SA-dependent but *NPR1*-independent pathways contribute to *cpr5/6*-mediated resistance. These pathways are, however, suppressed by the *ein2* and *jar1* mutations in *cpr/npr1/ein2* and *cpr/npr1/jar1* triple mutants. Importantly, a similar pathway appears to be operating in *RPS2*-mediated resistance against the bacterial pathogen *P. syringae* (19). Although *RPS2* function is not affected by the *npr1*, *ein2*, and *jar1* single mutations, it is compromised by the combined *npr1/ein2*, *npr1/jar1*, and *npr1/ein2/jar1* mutations. Since *RPS2* function is reduced to the same level in the SA-deficient *eds5* single mutant, two SA-dependent pathways must be required for function of this *R* gene: one *NPR1* dependent and one *EIN2/JAR1*-dependent.

The existence of crosstalk between SA and JA/ ET pathways is also strongly supported by global gene expression–profiling approaches. Two independent studies

(52, 130) identified sets of genes that are subject to antagonistic control by these pathways. Schenk et al. (130) also identified groups of genes that are coinduced or corepressed by SA, methyl jasmonate (MJ), or ET. Such genes that respond to more than one of the hormones tested may act downstream of important signal convergence points and will be valuable tools to dissect defense signaling networks in the future.

Glazebrook et al. (52) used global gene-expression profiling to predict in more detail the topology of the SA/JA/ET signaling network and to assign defense-signaling mutations either to SA-dependent or JA/ET-dependent pathways. Expression profiles suggested that *eds3* affects SA signaling, whereas *eds8* and *pad1* affect JA signaling. None of these mutations had been assigned to any of these pathways before. These predictions were experimentally confirmed. In addition, the existence of an as-yet unknown pathway operating downstream of pathogen recognition, which is SA and JA/ET independent but eventually converges with JA/ET-mediated signals, was postulated. This study impressively demonstrates that large-scale gene-expression profiling can effectively be used to make predictions on the topology of defense signaling networks.

Generally, the picture emerges that JA/ET-dependent pathways induce defense mechanisms protecting plants against necrotrophic pathogens, whereas ROI/SA-mediated responses are effective against biotrophic pathogens (97). Complex crosstalk between both signaling routes allows the plants to fine-tune their defense program and to respond to each type of pathogen with the most effective mixture of individual defense measures.

CONCLUSIONS: MIND THE TRIGGER

Profiling of global Avr/R gene-triggered gene-expression responses points to a certain degree of constitutive activity of *R*-gene pathways (102; T. Eulgem & J.L. Dangl, manuscript in preparation). In the absence of infection, mutants disrupted in distinct *R* pathways display reduced (or elevated) expression of defined gene sets. This result supports the notion that *R* activation is tightly controlled. Ectopic *R* expression can activate defense pathways in the absence of pathogen (32, 99, 107). After pathogen recognition, repression of these pathways is completely removed and they operate with maximal capacity, fully activating (or repressing) their target genes. The outcomes are production of potentially toxic secondary metabolites, programmed cell death, and the creation of a locally inhospitable environment for the pathogen. The consequences of *R* misexpression and misfiring, up to and including ectopic cell death, likely necessitate negative regulation of R-protein activation. This molecular regulatory concept is reflected back to the whole organism and ecological evolutionary level. A naturally occurring *rpm1* null allele is maintained in natural populations over very long time frames, suggestive of balanced polymorphism (56, 141), and the mere presence of functional *RPM1* in an otherwise isogenic background leads to an astounding 9% loss in seed production (149).

In the past few years, we have moved from understanding the basic genetic concepts behind *R* gene-mediated plant disease resistance into an exploration of how these processes are regulated at the molecular and biochemical levels. To quote U.S. Defense Secretary Donald Rumsfeld: "While there are more knowns now, there are still known unknowns and probably some unknown unknowns as well."

R-protein activation may be negatively regulated by intramolecular mechanisms, although how this is achieved and how they are activated will require more examples and more detailed biochemistry and structural biology. The role of accessory and partner proteins in R activation is just at its beginning, and will require both clever forward and reverse genetics approaches combined with proteome-based solutions. The guard hypothesis requires further testing, particularly as it relates to families of closely related *R* alleles. Signaling pathways leading from activated R proteins are being chipped away, and emerging concepts suggest that the resistant state is achieved by breaching quantitative activation thresholds, possibly driven by a central SA-driven positive feedback loop.

Among the many outstanding questions are: How do activated R proteins utilize components of basal resistance in order to amplify signal output? How do R proteins feed into the core feedback loop machinery to achieve this? Could it be that activated R proteins only associate transiently with their downstream targets? Answers to these questions will ultimately shape our understanding of how the plant immune system functions and evolves, and may one day lead us to the development of controlled, broad-spectrum resistant crops without the deleterious fitness costs.

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