Chapter A4

Recognition and Signal Transduction Associated with *R* Gene-mediated Resistance

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1.1 Introduction

Plants are constantly challenged by a wide array of pathogens, including viruses. For any specific plant species most viruses cannot surmount basal defenses that include physical barriers like a waxy layer covering the plant and post-transcriptional gene silencing (PTGS). However, in those instances when a virus is able to infect a plant, host survival relies on quick recognition of the invading virus and rapid signaling of a defense response. One form of resistance termed gene-for-gene type of resistance relies on the interaction of a plant *R* gene and a pathogen-encoded avirulence (*Avr*) gene. If a plant has a specific R protein that can recognize a pathogen Avr product, the plant will mount a defense response and thwart an infection. Therefore, plant R proteins have a dual role. Not only must they recognize a pathogen directly or indirectly, they must also initiate signaling that leads to a defense response. One of the earliest defense responses is the hypersensitive response (HR), a type of programmed cell death (PCD) that occurs at the pathogen's infection site. HR is correlated with the signaling of an *R*-gene-mediated disease resistance response and containment of the pathogen at the infection site (For details, see Chapter A5). Following HR, a systemic acquired resistance (SAR) response results in an enhanced resistance to further infection by a variety of pathogens. In this chapter we will discuss the major

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advances in understanding how R proteins recognize different viruses and the intricacies of the defense-signaling network that leads to HR and SAR.

1.2 R Genes and Recognition

Viral resistance genes

The disease resistance field has advanced quickly with the advent of cloned *R* genes. The tobacco *N* gene that confers resistance to *Tobacco mosaic virus* (TMV) was the first antiviral *R* gene cloned (Whitham et al. 1994). The cloning of *N* was a major breakthrough because it was one of the first R protein containing domains with a nucleotide binding site (NBS) and leucine-rich repeats (LRR). The NBS-LRR class represents the vast majority of *R* genes that confer resistance to viruses and other pathogens (Martin et al. 2003). The *N* gene belongs to a subclass of NBS-LRR genes that contain a Toll interleukin-1 receptor domain at the N-terminus (TIR-NBS-LRR). The only other cloned antiviral *R* gene that belongs to this subclass is the potato *Y-1*, which has 57% identity to the *N* gene (Vidal et al. 2002). Although the *Y-1* locus is known to confer resistance to *Potato virus Y* (PVY), it is yet unclear if this confers complete resistance.

The remaining cloned antiviral *R* genes belong to the NBS-LRR class, but contain a coiled-coiled domain at their N-terminus (CC-NBS-LRR). Potato *Rx1* and *Rx2* are two unlinked genes but they are functionally identical and confer extreme resistance to *Potato virus X (*PVX) without inducing HR (Bendahmane et al. 1999; Bendahmane et al. 2000). Tomato *Sw-5* confers resistance to *Tomato spotted wilt virus* (TSWV) and belongs to a seven-member gene family (Brommonschenkel et al. 2000). However, the pathogens recognized by the other family members are currently unknown. *Tm-2* and *Tm-2*^{2} provide resistance to *Tomato mosaic virus* (ToMV) (Lanfermeijer et al. 2003). Interestingly, the *Tm-2* allele of *Tm-2*^{2} is easily overcome by resistant strains of ToMV even though there are only 38 amino acid differences between the two alleles (Lanfermeijer et al. 2003).

The two antiviral *R* genes cloned from *Arabidopsis* include *HRT* from the Dijon-17 ecotype and *RCY1* from the C24 ecotype. *HRT* and *RCY1* confer resistance to *Turnip crinkle virus* (TCV) (Cooley et al. 2000) and the yellow strain of *Cucumber mosaic virus* (CMV-Y) (Takahashi et al. 2002) respectively. Interestingly, *HRT* and *RCY1* are allelic to *RPP8* from the Landsberg errecta ecotype that confers resistance to the fungus *Peronospora parasitica* (McDowell et al. 1998; Cooley et al. 2000). *RCY1* is 92.1% and 91.3% homologous to *HRT* and *RPP8* respectively (Takahashi et al. 2002). This is the first example of three alleles of the same gene conferring resistance to three different pathogens. This suggests that there must be

conserved mechanisms for both recognition and signaling by NBS-LRR genes.

The range of pathogens recognized by *RPP8/HRT/RCY1* family of *R* genes suggests plants may have evolved allelic series that can recognize, and therefore, confer resistance to a vast array of pathogens (Cooley et al. 2000). Alternatively, *R* genes may have diversified by duplication followed by a divergence of recognition. For example, *Gpa2* in potato is adjacent to *Rx1* but confers resistance to a nematode (van der Vossen et al. 2000).

Viral products recognized by R proteins

The diversity of recognition and the Tobamoviruses

R proteins recognize different types of virally encoded proteins. Tobamoviruses like TMV and ToMV encode three proteins: replicases, a movement protein (MP), and a coat protein (CP). *R* genes have evolved to recognize all three of these viral proteins. Additionally, R proteins appear to recognize viral proteins via protein-protein interactions rather than by detecting their function.

The N protein recognizes the helicase domain of the TMV replicases. *N* confers resistance to all tobamoviruses examined except the Ob strain of TMV (Tobias et al. 1982). Analysis of the Ob strain showed that amino acid changes in the helicase domain of the replicase proteins allowed it to surmount *N*-mediated resistance (Padgett and Beachy, 1993; Padgett et al. 1997). Expression of the helicase domain in plants containing the *N* gene induces resistance response proving that the helicase domain alone is necessary and sufficient to elicit an *N*-mediated response (Erickson et al. 1999). Furthermore, the N protein might recognize TMV helicase via protein-protein interactions because ATPase activity of the helicase domain is not required for recognition (Erickson et al. 1999).

The allelic genes *Tm-2* and *Tm-2*^{2} recognize the MP of ToMV (Meshi et al. 1989; Weber et al. 1993). Resistance breaking strains contain mutations in the variable C-terminus of the MP that is dispensable for ToMV movement or replication (Gafny et al. 1992). This suggests that Tm-2 alleles do not recognize the function of the MP, but rather, the recognition probably occurs via protein-protein interactions.

The tobacco *N'* gene has not been cloned, but extensive studies have shown that it recognizes the TMV CP (Saito et al. 1989). Domain swaps between the TMV-L strain that induces HR and the TMV-OM strain that does not induce HR has shown that the CP of TMV-L strain is necessary to elicit a response in the presence of N'. Deletion analysis shows that the entire CP except 13 amino acids at the C-terminus are needed for recognition by N'

(Saito et al. 1989). It appears that proper formation of the tertiary structure of assembled coat proteins is necessary for N' to recognize TMV (Toedt et al. 1999).

Recognition of coat proteins

The CP is most common viral product recognized by cloned R proteins. For example, antiviral *R* genes such as *Rx1, Rx2, N', HRT* and *RCY1* all recognize CP.

Mutations in the CP of resistance breaking strains of PVX were responsible for their evasion of *Rx1*-mediated recognition. The protein, and not the RNA of CP, is recognized by Rx1 because sequence differences that did not change amino acids were unnecessary for PVX resistance breaking strains to evade Rx1 detection (Kohm et al. 1993). To determine if the CP alone is sufficient to elicit resistance by Rx1, recombinant TMV expressing the PVX CP was expressed in protoplasts containing *Rx1*. TMV normally can infect and replicate in protoplasts containing *Rx1*. However, TMV expressing the PVX CP could not replicate in protoplasts containing *Rx1* (Bendahmane et al. 1995). Therefore the PVX CP is sufficient to elicit an *Rx1*-mediated resistance response to PVX.

The *R* genes *HRT* and *RCY1* recognize the CP of TCV and CMV respectively (Zhao et al. 2000; Takahashi et al. 2001). The *HRT* and *RCY1* genes are highly homologous, but the CPs they recognize contain no sequence homology. Domain swaps between CMV-Y that induces an *RCY1*-mediated defense and CMV-B2 that cannot be detected by RCY1, suggest that the CP is necessary for recognition (Takahashi et al. 2001). However, it remains unknown if the CP alone is sufficient for recognition by *RCY1*, or if other CMV proteins are required.

Recognition of proteases

The *Ry* gene has not been cloned, but it confers a durable, extreme resistance to PVY. The nuclear inclusion a protease (NIaPro) from PVY can elicit *Ry*-mediated resistance response. Although an intact protease site is necessary for NIaPro to elicit a defense response, the protease activity of NIaPro is not sufficient for the elicitation of *Ry*-mediated resistance (Mestre et al. 2003). A mutant with close to wild-type protease activity is unable to elicit a defense response, indicating that NIaPro is either recognized via a protein-protein interaction or has another protease activity that cleaves a host protein.

1.3 The mode of recognition

The guard hypothesis

How do R proteins recognize specific Avr products from viruses? Direct interaction between an elicitor from the virus and a corresponding antiviral R protein has not been shown. However, in a few cases direct interactions between R proteins and Avr ligands has been demonstrated for interactions between plants and non-viral pathogens (Scofield et al. 1996; Tang et al. 1996; Jia et al. 2000; Deslandes et al. 2003). Since most cloned R proteins fail to interact directly with cognate Avr proteins, alternative hypotheses have been proposed. The most popular model is the "guard hypothesis" (Dangl and Jones, 2001). The guard hypothesis states that R proteins act as "guards" that monitor key host cellular factors called "guardees" that are modified by a pathogen's Avr product. R proteins recognize the pathogen by perceiving a change in the status of the cellular factor. The guard hypothesis may explain some non-viral host-pathogen interactions. For example, in the interactions between avirulent strains of *Pseudomonas syringae* and *Arabidopsis thaliana*, two different *R* gene products guard the cellular protein RIN4. Rpm1 recognizes hyperphosphorylation of RIN4 caused by AvrRpm1 or AvrB and then induces a resistance response (Mackey et al. 2002). Another bacterial R protein, RPS2, recognizes the rapid degradation of RIN4 (Axtell and Staskawicz, 2003; Mackey et al. 2003). Likewise, RPS5 recognizes the proteolytic cleavage of PBS1 kinase by the bacterial protein, AvrPphB (Shao et al. 2003).

There is currently one virus-plant interaction that supports the guard hypothesis as the model for viral recognition. A yeast two-hybrid screen conducted with the TCV CP yielded the TCV-interacting protein (TIP) from *Arabidopsis* (Ren et al. 2000). The interaction between TIP and TCV-CP is necessary for *HRT*-mediated resistance and therefore, HRT may be guarding the host protein TIP. However, it has not been directly shown that HRT is guarding TIP. An alternative hypothesis is HRT is guarding a protein that binds to TIP or is regulated by TIP. TIP belongs to the NAC family and has the ability to induce transcription in yeast. TIP-regulated transcription of genes may change when TCV CP binds TIP, and HRT might indirectly recognize that change in transcription.

R protein-containing complexes

The guard hypothesis suggests that the host-pathogen interaction is more likely an interaction between the Avr protein and a host recognition complex. This complex must be able to recognize the pathogen and signal a defense response. Complex levels and activation of signaling must be tightly regulated and the recognition complex must be poised to perceive and respond to pathogens. To understand the function of protein complexes during disease resistance, we must determine the components of the complex, how the complex forms, and how the complex is activated to signal defense.

The Hsp90-Sgt1-Rar1 complex

Recently, a few proteins that may belong to R protein-containing signaling complexes have been discovered. The 90 kDa heat shock protein (Hsp90) was the first protein discovered to interact directly with an antiviral R protein. The LRR domain of N directly interacts with Hsp90 in a yeast two-hybrid assay (Liu et al. 2004b). In addition, Hsp90 coimmunoprecipitates with the full length N protein *in vivo* (Liu et al. 2004b). Two independent research groups have shown that silencing of *Hsp90* compromises *N*-mediated resistance to TMV (Lu et al. 2003; Liu et al. 2004b). *Hsp90* has also been shown to be necessary for the function of the *R* genes *Rpm1*, *RPS2*, and *Pto* that confer resistance to different strains *Ps. syringe* and *Rx1*, which confers resistance to PVX (Hubert et al. 2003; Lu et al. 2003; Takahashi et al. 2003).

The exact role of *Hsp90* during disease resistance is unknown. Hsp90 is a highly conserved eukaryotic ATP-dependent chaperone that facilitates protein folding and activation of proteins (Picard, 2002). Therefore, it is possible that Hsp90 has a non-specific role during protein folding of R proteins and other components involved in *R* gene-mediated resistance (Shirasu and Schulze-Lefert, 2003). The Rx1 protein levels decrease when *Hsp90* is silenced, and therefore Hsp90 may control the stability of Rx1 (Lu et al. 2003). However, two pieces of evidence suggest that Hsp90 plays a more direct role during disease resistance. Rare Hsp90 mutants in *Arabidopsis* do not have severe morphological phenotypes, but do have an attenuated *Rpm1*-mediated resistance response (Hubert et al. 2003). Additionally, Hsp90 directly interacts with the resistance protein N and other components of disease resistance signaling.

Hsp90 interacts with two defense signaling components, Rar1 and SGT1 (Takahashi et al. 2003; Liu et al. 2004b)**.** *Rar1* was originally identified as a gene required by multiple *Mla* resistance genes in barley (Shirasu et al. 1999). Silencing of *Rar1* in *NN* plants compromises *N*-mediated resistance to TMV (Liu et al. 2002a)**.** SGT1 interacts directly with RAR1 and Hsp90 and

silencing of *SGT1* compromises *Rx1*-mediated resistance to PVX (Peart et al. 2002b) and *N*-mediated resistance to TMV (Liu et al. 2002b; Peart et al. 2002b)**.** SGT1 also plays a second role downstream of recognition during defensive signal transduction (see above). It is tempting to speculate that Hsp90 is a chaperone that regulates the folding and formation of an R protein-containing complex, and recruits SGT1 and Rar1 as co-chaperones. Thus, a possible role for the Hsp90-SGT1-Rar1 complex might be to finely adjust the abundance or activation status of R protein-containing complexes (Hubert et al. 2003).

Intramolecular interactions of R proteins

Changes in the recognition complex may be initiated by changes in intramolecular interactions of R protein domains. There is convincing evidence that such interactions occur in R proteins that belong to the CC-NBS-LRR subclass. The CC-NBS domain and the LRR domain of Rx1 physically interact with each other (Moffett et al. 2002). This interaction is disrupted only in the presence of an *Rx1*-eliciting PVX CP but not in the presence of a non-eliciting PVX CP. This suggests that recognition may occur by the viral elicitor by directly or indirectly disrupting the intramolecular domain interactions of R proteins. However, a more likely model places the disruption of intramolecular interactions directly downstream of recognition. The abrogation of domain interactions may alter the components of the R protein complex by exposing or activating signaling domains, such as the CC or NBS, which then recruit signaling components to the complex. Alternatively, a signaling component that constitutively belongs to the complex may be activated and released to initiate signaling pathways.

Signal transduction

After a virus is recognized, the function of an R protein complex must switch from recognition to signal transduction. Intramolecular interactions, activation of the NBS domain, and changes in signaling components that may associate with the CC or TIR domain and LRR domain have all been implicated during early signaling. However, their precise roles during the initiation of signal transduction remain elusive. On the contrary, researchers have made major advances in identifying crucial small signaling molecules, defense-related signaling pathways, and shared signaling components.

Early signaling in the hypersensitive response

Reactive Oxygen Intermediates

The HR is a type of localized cell death that occurs at the infection site of viruses and is correlated with, but not always required for, the restriction of viruses (For a detailed discussion, see Chapter A5). HR is dependent on the production of reactive oxygen intermediates (ROIs), mainly in the form of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) (Grant and Loake, 2000). The formation of ROIs upon pathogen infection has been identified during infection with bacteria, fungi, and viruses.

The production of ROIs is biphasic. The first phase is a small burst that happens within minutes of infection. This is induced by an infection with both virulent and avirulent pathogens. For example, the induction of ROIs by the TMV CP is independent of *N* (Allan et al. 2001). The second phase is stronger, more persistent, and is correlated with disease resistance. Doke and Ohashi (1988) were the first to determine that ROIs play an important role during *R* gene-mediated resistance to viruses. They discovered an O_2^- generating system that is activated during *N*-mediated response to TMV and that occurs as a burst (Doke and Ohashi, 1988). Moreover, the generation of O_2^- was dependent on Ca^{2+} and coupled to NADPH, which suggest O_2^- may be produced by an NADPH oxidase.

O_2^- is converted to H_2O_2 spontaneously or actively by superoxide dismutase (SOD) (Lamb and Dixon, 1997). Alternatively, H_2O_2 can accumulate when antioxidants such as ascorbate peroxidase, catalase, and carbonic anhydrase are inhibited (Chen et al. 1993; Durner and Klessig, 1995; Slaymaker et al. 2002). A third source of H_2O_2 may come from the extracellular space, also known as the apoplast. Pathogen-induced H_2O_2 moves apoplastically and may be generated by cell wall-localized peroxidases or from polyamine oxidases (Allan and Fluhr, 1997; Yoda et al. 2003). Polyamine oxidases convert polyamines to H_2O_2 by oxidative deamination (Yoda et al. 2003). During TMV-induced HR the polyamine, spermine, is up-regulated 20-fold in the apoplast (Yamakawa et al. 1998).

H_2O_2 is necessary for the regulation of HR. A 2- to 4-fold over-expression of the catalase gene *CTA1* decreases the level of H_2O_2, which subsequently results in larger TMV-induced HR lesions (Talarczyk and Hennig, 2001). However, the targets of H_2O_2 that induce signal transduction are largely unknown. One possibility is H_2O_2 may control Ca^{2+} influx. H_2O_2 induces a dose-dependent increase in cytoplasmic Ca^{2+} that plays an important role during HR (Levine et al. 1994). Alternatively, H_2O_2 may not control HR by directly binding to protein effectors, but rather, may affect

signaling pathways that are sensitive to changes in the cellular redox state. H_2O_2 induces the expression of glutathione-S-transferase and glutathione peroxidase (Levine et al. 1994). These enzymes quickly change the cellular redox state to a more reducing environment. Recently, changes in the cellular redox state were shown to be crucial for mounting a defense response. A reduced cellular environment causes the important signaling component, NPR1, to become monomeric and move into the nucleus (Mou et al. 2003). Once it is there, it binds to TGA transcription factors that induce the expression of defense-related genes (Despres et al. 2003; Mou et al. 2003).

Nitric oxide

The production of nitric oxide (NO) is also biphasic, and occurs at approximately the same time as the production of ROIs (Delledonne et al. 1998). It is thought that NO and ROIs cooperate to signal a HR (Delledonne et al. 1998). NO, like H_2O_2, is an excellent candidate molecule for cell-to-cell signaling. Even though NO is highly reactive with oxygen, it still has a half-life of a few seconds and has the ability to diffuse across membranes (Beligni et al. 2002; Neill et al. 2002).

In mammals, NO signals through a cyclic GMP (cGMP)-dependent pathway. In tobacco, NO and cGMP induce *PAL* and *PR-1*, which are early and late markers of defense respectively (Durner et al. 1998). NO increases the abundance cGMP, which may be formed by guanylate cyclase. The increase in *PAL* expression can be inhibited by LY8358 and ODQ, two inhibitors of mammalian guanylate cyclase. Downstream of cGMP, cyclic ADP ribose (cADPR) functions during Ca^{2+} regulation (Denninger and Marletta, 1999). Addition of cGMP and cADPR to tobacco leaf discs causes a synergistic increase in PAL and PR-1 expression. These data suggest that NO signals partially through a cGMP signaling pathway (Durner et al. 1998). Alternatively, NO may induce disease resistance response through nitrosylation of redox-sensitive amino acids, such as cysteine or tyrosine, or by reacting with transition metal centers (Stamler et al. 2001; Romero-Puertas et al. 2004). However, the role of nitrosylation in plants is currently unclear.

Salicylic acid and the hypersensitive response

In addition to ROIs and NO, SA has been implicated in HRs to both viral and non-viral pathogens. SA is not sufficient for the induction of HR because supplying it exogenously does not cause a HR. However, SA may be necessary to regulate the timing and extent of the HR.

During the HR, SA forms a gradient, with SA accumulating to high levels at the center of the HR lesions, moderate levels at the lesion borders, and low levels in healthy tissue (Enyedi et al. 1992). This accumulation of SA during a TMV-induced HR in *NN* plants is biphasic (Mur et al. 1997). There is a 10-fold increase in the pre-necrotic phase and a 50-fold increase in the necrotic phase. Constitutive expression in transgenic plants of the bacterial *nahG* gene, which encodes the SA-degrading enzyme, salicylate hydroxylase, decreases accumulation of SA in plants. This results in the attenuation of SA-mediated signaling. Thus, in transgenic NN genotype tobacco plants harboring *nahG* constructs driven by CaMV 35S promoters, TMV induces larger sized HR lesions that may eventually lead to a spreading necrosis phenotype (Gaffney et al. 1993; Mur et al. 1997). Interestingly, transgenic *NN*-genotype tobacco plants containing a *nahG sequence* under the tobacco *PR-1a* promoter (which is itself SA-responsive) only lose the second phase of SA accumulation. These plants have similar size lesions to those on plants which do not contain the *N* transgene. A greater increase in lesion size was seen in transgenic plants where the asparagus *AoPR1* promoter was used to drive expression of salicylate hydroxylase (Mur et al. 1997). This promoter is responsive to ROI, not SA, and is active during the pre-necrotic phase of the HR. Therefore, it appears that SA accumulation during the early, pre-necrotic phase of the HR is the most critical for controlling *N*-mediated restriction of TMV spread (Mur et al. 1997).

SA also plays a direct role in resistance to TCV during the *HRT*-mediated HR in Arabidopsis. In *NahG*-transgenic *HRT*-containing plants, resistance to TCV was completely lost in all the plants tested (Kachroo et al. 2000). The cell death associated with HR was undetectable by tryphan blue (Kachroo et al. 2000). Therefore, unlike in the TMV-induced HR in tobacco, during the TCV-induced HR in Arabidopsis both cell death and resistance require an SA-dependent signaling pathway or pathways. This is one of many examples of varying requirements and functions of signaling pathways during resistance to different viruses and/or in different host species.

Varying roles of jasmonic acid and ethylene

Most viruses require SA-dependent signaling pathways. However, the requirement of ethylene (ET) and jasmonic acid (JA) during *R* gene-mediated resistance to viruses is more complex and variable. The crosstalk between ET-, JA- and SA-dependent signaling pathways can have synergistic or antagonistic effects on each other. ET and JA are secondary signaling molecules that function in microbial defense, wounding, and insect attack. During SAR, they induce the expression of specific genes that are not induced by SA. JA induces the expression of *thionin 2.1 (Thi2.1)* and both

JA and ET induce the expression of *Plant Defensin 1.2* (*PDF1.2*) (Kunkel and Brooks, 2002).

Ethylene signalling

Resistance to TMV requires an ET-dependent signaling pathway. The *Arabidopsis* mutant, *ctr1*, undergoes a constitutive triple response to ethylene and functions downstream of ethylene receptors (Kieber et al. 1993). Silencing *NbCTR1* causes a constitutive ethylene response and results in a rapid initiation of TMV-induced HR in *NN*-transgenic *N. benthamiana* plants (Liu et al. 2004a). Analysis of ethylene-insensitive transgenic tobacco (Tet^r) plants indicates ethylene is necessary for SAR and may be necessary for creating or moving the mobile signal necessary for SAR; however, it is unnecessary for sensing the mobile signal (Knoester et al. 1998).

In *Arabidopsis RCY1*-mediated resistance to CMV also requires ET signaling pathways. Only 8% of the *RCY1*-containing ethylene insensitive mutants, *etr1-3* or *ein2-1*, were susceptible to CMV-Y infection (Takahashi et al. 2004). When NahG depleted SA in RCY1 plants, 16% of the plants were susceptible. *RCY1* plants with both *NahG* and *etr1-2*, resulted in 57% susceptibility to CMV-Y (Takahashi et al. 2004). This suggests SA and ET may function synergistically. Furthermore, a third pathway or mechanism must exist to explain why almost half of the plants remained resistant to CMV-Y.

In contrast to *N* and *RCY1*, *HRT*-containing *etr1-1* plants were completely resistant to TCV, and therefore, the ET signaling pathway is not required for resistance mediated by this gene (Kachroo et al. 2000).

Jasmonic acid signalling

Jasmonic acid signaling is required for *N*-mediated resistance to TMV. Silencing of *CORONATINE-INSENSITIVE1* (*COI1)* compromises *N*-mediated resistance to TMV in *NN*-transgenic *N. benthemiana* (Liu et al. 2004a). Furthermore, the levels of JA and its metabolic precursor OPDA (cis-12-oxophytodienoic acid) increase in *NN* tobacco plants infected with TMV (Dhondt et al. 2000).

In contrast, the *coi1-1* mutation in *HRT Arabidopsis* plants did not affect resistance to TCV, suggesting JA is not required (Kachroo et al. 2000). *RCY1*-mediated resistance does not require JA, but rather, JA and SA signaling pathways mutually antagonize each other. JA signaling suppresses the SA-induced expression of *PR-1* and *PR-5* during *RCY1*-mediated resistance to CMV-Y (Takahashi et al. 2002). Conversely, SA signaling suppresses JA-induced expression of *PDF1.2* and *HEL*, two known markers for JA signaling (Takahashi et al. 2002). The varying requirements for ET,

JA, and SA during *RCY1*- and *HRT*-mediated resistance are surprising because these genes are highly similar. One explanation is the requirement for different signaling pathways diverged as the specificity of *RCY1* and *HRT* evolved. Alternatively, all three pathways may be initiated to the same degree by *RCY1* and *HRT*, but the effect of the downstream resistance mechanisms on movement or replication of CMV-Y or TCV may vary.

Signals needed for induction of systemic acquired resistance

SAR confers long-lasting resistance to secondary infections of a wide variety of pathogens, including viruses, bacteria, oomycetes, and fungi (Durrant and Dong, 2004). JA and ET signaling pathways may be necessary for SAR during defense to a variety of pathogens; however, their role during virus-induced SAR is unclear. Many signaling pathways and components have roles during both HR and SAR. For example, SA has varying importance during HR, but has a well-established function during SAR. The ability of SA to induce SAR to viruses was exhibited when exogenously supplied SA in the form of aspirin (acetyl-SA) was shown to reduce the size of HR lesions by 95% during an N-mediated defense response to TMV (White, 1979). One of the hallmarks of SAR is the induction of a set of pathogenesis-related (PR) proteins (Durrant and Dong, 2004). The PR proteins with chitinase and β1,3-glucanase activities have anti-fungal and anti-bacterial properties (Bowles, 1990), but the known PR proteins have not been shown to have anti-viral activities.

SA signaling through the NPR1-dependent pathway

The *Arabidopsis* mutants *npr1* (non-expressor of PR-1), *nim1* (noninducible immunity1), and *sai1(*salicylic acid-insensitive1) are allelic mutations in the *NPR1* gene (Durrant and Dong, 2004). SA or avirulent pathogens fail to induce SAR in *npr1* mutants (Durrant and Dong, 2004). SA induces the expression of PR proteins through an *NPR1*-dependent pathway during both HR and SAR (Durrant and Dong, 2004). SA induces the nuclear localization of NPR1, where it binds to TGA transcription factors that increase the expression of *PR* genes or other defense genes (Durrant and Dong, 2004). The SA-binding protein 2 (SABP2) gene encodes a lipase protein that may be the receptor for SA signaling through the *NPR1*-dependent pathway, because *SABP2*-silenced tobacco plants have a similar phenotype to the Arabidopsis mutant *npr1* mutant. *SABP2*-silenced plants had 41% larger HR lesions, failed to induce SAR, and had a reduced up-regulation of PR-1 expression compared to the wild-type *NN*-genotype tobacco plants (Kumar and Klessig, 2003). The lipase activity of SABP2 is

activated by SA binding and the expression of SABP2 is induced in TMV-infected *NN* plants (Kumar and Klessig, 2003).

R genes for viral recognition have varying requirements for NPR1. NPR1-dependent signaling is necessary for *N*-mediated resistance to TMV because silencing of *NPR1* in *NN*-transgenic *N. benthamiana* plants resulted in a loss of *N*-mediated resistance to TMV (Liu et al. 2002a). *Arabidopsis* plants with the HRT gene and with the *npr1-1* or *npr1-5* mutations had a delayed HR and decreased levels of PR-1, but resistance to TCV is not compromised (Kachroo et al. 2000). Since NahG plants that cannot accumulate SA remained susceptible to TCV, SA must also signal through a pathway that is independent of NPR1. Furthermore, resistance to turnip vein clearing virus (TVCV) can be induced by SA in non-HR responding *Arabidopsis npr1* mutants (Wong et al. 2002).

SA signaling through the SHAM sensitive pathway

An NPR1-independent pathway was recently discovered to be specifically required for resistance to viruses. Salicylhydroxamic acid (SHAM) blocks SA-dependent resistance to TMV in tobacco (Chivasa et al. 1997). Remarkably, SHAM does not inhibit resistance to the bacterial pathogen *Erwinia carotovora*, or the fungal pathogen *Botrytis cinerea*, suggesting that the SHAM sensitive pathway is specific to defense against viruses (Chivasa et al. 1997). The SHAM-sensitive pathway does not induce PR proteins, and consequently, is independent of the *NPR1*-dependent pathway.

SHAM is an inhibitor of alternative oxidase (AOX) as well as an inhibitor of SA-induced resistance to TMV in tobacco (Chivasa et al. 1997). This suggests increases in AOX should induce resistance to TMV. *AOX* transcripts do increase when TMV elicits an *N*-mediated response (Chivasa and Carr, 1998). The metabolic inhibitors antimycin A (AA) and potassium cyanide (KCN) inhibit electron transfer in the cytochrome pathway, which results in an increase in *AOX* transcript levels. This indirect induction of *AOX* correlates with resistance to TMV (Chivasa and Carr, 1998). AA and KCN induce TMV resistance, possibly through AOX, but do not cause an increase in PR-1 (Chivasa and Carr, 1998; Murphy et al. 1999). Additionally, KCN is able to restore the loss-of-resistance caused by a depletion of SA by the SH-L transgene. SHAM, which has the opposite affect of KCN, prevents KCN from restoring resistance (Chivasa and Carr, 1998). Therefore, KCN and SHAM affect the same pathway, possibly through AOX. However, the role of AOX is still unclear because KCN, AA, and SHAM are pharmacological reagents that affect other proteins. Stable over-expression or knockdown lines of AOX will clarify its function during defense (see Chapter 15 by Handford and Carr).

Signaling components and downstream effectors of the SHAM-sensitive pathway are currently unknown. The SHAM-sensitive pathway is necessary for resistance to TMV in tobacco, but it is unclear if it necessary for resistance to other viruses or if it functions in other plant species (see Chapter 6 by Handford and Carr). However, the discovery of the SHAM-sensitive pathway is quite important because it is the first biologically significant virus-specific pathway that defines a new mode of SA-dependent signal transduction.

Spermine-induced signaling pathway

The possible role of polyamines (PAs) during disease resistance to viruses is often overlooked. The most abundant PAs are putrescine (Put), spermidine (Spd) and spermine (Spm) (Janne et al. 2004). They are poly-cationic compounds with a flexible carbon backbone that have the ability to associate with negatively charged compounds, such as nucleic acids, acidic phospholipids, and proteins. In *NN* plants infected with TMV, Spm levels increase by 20 fold suggesting that PAs may play a role in *N*-mediated resistance to TMV (Yamakawa et al. 1998). This increase only occurs in the intercellular spaces and was not detected in whole cellular leaf extracts. The protein abundance levels of PR-1, PR-2, PR-3, and PR-5 increase in response to Spm, SA, and TMV, suggesting Spm may be necessary for SAR. Interestingly, exogenously supplied Spm enhances HR formation in a dose-dependent manner and therefore enhances *N*-mediated resistance to TMV.

SA and Spm may function in separate pathways because SA is unable to cause increases in Spm levels and Spm is unable to cause increases in SA levels. Additionally, the tobacco peroxidase genes, tpoxC1 and tpoxN1, are induced by Spm, but not by SA (Hiraga et al. 2000). Exogenously supplied Spm causes an increase in expression of *HIN1*, *HIN9*, and *HIN18* and this is unaffected in *NahG*-transgenic plants (Yamakawa et al. 1998). Similar upregulation of *HIN1* expression was observed in *NN*-genotype tobacco plants infected with TMV. Spm or TMV specifically induces the increase in HIN1 because other PAs, SA, ET, and JA fail to induce *HIN1* expression. Spm may be important for resistance to other viruses. In Arabidopsis, the up-regulation of *HIN10* during *RCY1*-mediated resistance to CMV-Y is completely independent of SA (Zheng et al. 2004).

Downstream signaling components

Early downstream signaling components

The CC and TIR domains of R genes are structurally different and may signal through different signaling pathways. Many of the TIR-NBS-LRR class of R proteins signal through an *EDS1*-dependent pathway and the CC-NBS-LRR class of R proteins signal through the *NDR1*-dependent pathway (Aarts et al. 1998). *EDS1* is necessary for the function of TIR-NBS-LRR subclass antiviral R protein N (Liu et al. 2002a; Peart et al. 2002a) but not for the CC-NBS-LRR subclass protein Rx1 (Peart et al. 2002a).

The *Arabidopsis* mutant *ndr1-1* has compromised resistance to both bacteria and fungi, but its requirement during virus resistance has not been investigated. *RPP8*, which belongs to the same family as *RCY1* and *HRT* does not require *EDS1* or *NDR1* (McDowell et al. 2000). Therefore, a pathway that is independent of *EDS1* and *NDR1* must exist. *RPP8* has 98% homology to *HRT* and 93% homology to *RCY1* in the CC domain, suggesting that the two antiviral *R* genes products may signal through a third, unknown signaling component. It is possible that *HRT* and *RCY1* do not need *NDR1* but may need one of its homologues. There are 45 *NDR1/HIN1-like* (*NHL*) genes in *Arabidopsis* (Zheng et al. 2004). Interestingly, *NHL10* is highly up-regulated during an RCY1-mediated response to CMV-Y but not during the susceptible response to CMV-B2. Mutants in *EDS1*, *NDR1*, and *NHL10* will have to be analyzed further to determine if *HRT* or *RCY1* signal through one or more of these signaling components.

MAPK cascades

Mitogen-activated protein kinase (MAPK) cascades play roles diverse in plant processes that include cytokinesis, phytohormone signaling, wound responses, osmotic stress, and pathogen resistance (reviewed in Zhang and Klessig, 2001). A MAPK cascade proceeds via a hierarchy of protein kinases in which a MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK)by phosphorylation, which in turn, activates a MAPK by phosphorylation. During *N*-mediated resistance to TMV two MAPKs, SA-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), are activated (Zhang and Klessig, 1998a; Zhang and Klessig, 1998b). Recently it was discovered that a naturally- occurring diterpene, (11E, 13*E*)-lambda-11,13-diene-8α, 15-diol, known as WAF-1 may be the upstream activator of WIPK and SIPK (Seo et al. 2003). Exogenously supplied synthetic and natural WAF-1 activate WIPK and SIPK independently of SA signaling (Seo et al. 2003). WAF-1 levels increase rapidly upon TMV infection in *NN* plants, suggesting WAF-1 may be the endogenous signal for WIPK and possibly SIPK during disease resistance (Seo et al. 2003).

The biological role of SIPK and WIPK function during viral resistance was investigated by over-expression and silencing of *SIPK* and *WIPK*. Silencing *SIPK* and *WIPK* by PVX-based virus-induced gene silencing (VIGS) resulted in an attenuation of *N*-mediated resistance to TMV (Jin et al. 2003). Interestingly, even though both are necessary for defense, over-expression of *SIPK*, but not *WIPK*, leads to HR-like cell death. Silencing of *WIPK* leads to a loss of *N*-mediated resistance but has no effect on the HR**.** Directly upstream of *SIPK* and *WIPK* is *NtMEK2*, a MAPKK (Yang et al. 2001; Jin et al. 2003). A constitutively active form of NtMEK2, $NtMEK2^{DD}$, causes an activation of SIPK and WIPK and leads to HR-like cell death (Yang et al. 2001). Silencing *NtMEK2* also causes an attenuation of *N*-mediated resistance to TMV (Jin et al. 2003). The MAPKKK upstream of *NtMEK2* and the downstream target of *SIPK* or *WIPK* are currently unknown.

A complete MAPK cascade involving The *NPK1*-*MEK1*-*NTF6* has been shown to be necessary for *N*-mediated resistance to TMV (Liu et al. 2004a)**.** Silencing of the MAPKKK, *NPK1*, by VIGS compromises the function of the *N* gene (Jin et al. 2002). Silencing the downstream MAPKK, *MEK1*/*NQK1*, and the MAPK, *NTF6*/*NRK1*, by VIGS cause a loss of *N*-mediated resistance to TMV (Liu et al. 2004a).

Transcription factors

TGA, MYB, and WRKY families of transcription factors have been implicated in disease resistance. Activation of TGA by NPR1 is necessary for SA-dependent resistance to non-viral pathogens (Durrant and Dong, 2004). The triple mutant of *tga2-1, tga5-1,* and *tga6-1* was unable to induce the expression of PR genes in response to an SA analog, 2,6 dichloroisonicotinic acid (INA) (Zhang et al. 2003). However, single and double mutants were still responsive to INA suggesting that *TGA2*, *TGA5*, and *TGA6* function redundantly in signaling NPR1-dependent regulation of *PR* genes (Zhang et al. 2003). Silencing of *TGA1a, TGA2.1, TGA2.2,* and *TGA6* by VIGS did not compromise *N*-mediated resistance to TMV (Liu, Y., Schiff, M., and S.P.D-K, unpublished results). However, silencing multiple TGA factors by mixed infection resulted in a partial loss-of-resistance to TMV. Therefore, it is likely that *N*-mediated resistance responses are *NPR1*-dependent and function through TGA transcription factors. Further research must be conducted to conclusively determine the role of TGA transcription factors during viral resistance.

MYB1 expression is induced by SA and during the *N*-mediated response to TMV in tobacco (Yang and Klessig, 1996). The transcription factor MYB1 has been shown to bind to the sequence GTTTGGT in the promoter of *PR-1a*. *MYB1* plays a biologically significant role during *N*-mediated defense because silencing of *NbMYB1* attenuates *N*-mediated resistance to TMV in *N*-transgenic *N. benthamiana* using VIGS (Liu et al. 2004a).

WRKY transcription factors play a crucial role in regulating multiple defense response genes (Eulgem et al. 2000). WRKY transcription factors bind to the W-box ((T)TGAC(C/T)) sequence found in the promoters of various genes including *PR-1*, *PR-2*, and *PR-3* (Eulgem et al. 2000). Overexpression of *WRKY70* induces the constitutive expression of PR proteins (Li et al. 2004). *NtWRKY3* and *NtWRKY4* are highly induced by SA and during the *N*-mediated response to TMV (Chen and Chen, 2000), while the level of *WRKY1* transcript increases to a lesser degree and the level of *WRKY2* transcript does not change (Yang et al. 1999). VIGS of *WRKY1*, *WRKY2*, or *WRKY3* in *NN-transgenic N. benthamiana* plants compromises *N*-mediated resistance to TMV but has no effect on HR (Liu et al. 2004a). This is the first biological evidence that WRKY factors are necessary for viral resistance. However, the function of other members of this numerous family in disease resistance remains to be investigated.

Expression of *WRKY* transcription factors are up-regulated after the activation of SIPK and WIPK. Additionally, SIPK and WIPK induce W-box binding activity of unidentified WRKYs *in vivo* (Kim and Zhang, 2004). This suggests that *WRKYs* are downstream of the *MEK2-SIPK/WIPK* cascade. WRKYs are not phosphorylated, and therefore, there must be additional unknown components between SIPK/WIPK and WRKYs (Kim and Zhang, 2004). Additionally, WRKYs may function by regulating *NPR1* gene expression. The promoter of *NPR1* has three W-box domains that SA-induced WRKY transcription factors specifically bind to (Yu et al. 2001). However, these specific WRKYs have not been identified.

Protein degradation in defensive signaling

Degradation by the 26S proteosome

SGT1 has a second function during disease resistance. In yeast, SGT1 is a conserved component of the ubiquitin ligase SCF (SKP1, Cullin/F-box proteins) complex (Kitagawa et al. 1999)**.** SCF complexes recruit specific proteins and catalyze covalent attachment of ubiquitin. Often, ubiquitinated proteins are targeted for subsequent degradation via the 26S proteasome (Deshaies, 1999). The SGT1-SCF complex may function by targeting regulatory proteins for degradation via the 26S proteosome. For example,

the ubiquitin ligase $SCF^{EBF1/EBF2}$ directs the proteolysis of the EIN3 transcription factor that is both necessary and sufficient for the activity of the ethylene signaling pathway (Guo and Ecker, 2003). The SCF^{COI1} complex regulates JA sensitive genes by controlling protein degradation of histone deacetylases (Devoto et al. 2002). Interestingly, NbSGT1b interacts directly with NbSKP1 of the SCF complex and silencing either of these genes in *N. benthamiana* compromises *N*-mediated resistance to TMV (Liu et al. 2002b).

Recently it was shown that SCF^{COI1} complex, like other plant SCF complexes, is regulated by the COP9 signalosome (CSN) (Feng et al. 2003). The CSN regulates the SCF complex activity by modulating cycles of addition and removal of the ubiquitin-like protein NEDD8 to the SCF subunit, Cullin1 (Deshaies, 1999). Silencing the genes encoding the CSN subunits, *CSN3* and *CSN8*, results in a loss of *N*-mediated resistance to TMV (Liu et al. 2002b). Furthermore, SGT1 associates with the CSN, which provides additional evidence that SGT1 is involved in SCF complex regulation by the CSN (Azevedo et al. 2002; Liu et al. 2002b). In conclusion, silencing of many different components of the ubiquitin-proteosome degradation machinery has resulted in a loss of *N*-mediated resistance to TMV. Since F-box proteins determine the substrate specificity of SCF complexes, it will be important to determine which F-box proteins are necessary for disease resistance and the identity of the F-box's substrates that are subsequently targeted for degradation.

Degradation by caspase-like proteins

The destruction of mammalian cells during PCD is often mediated by caspases that specifically cleave substrates (Zhivotovsky, 2003). There are no known homologues to mammalian caspases in plants but it appears that proteins with caspase-1 or -3 activity play crucial role during the initiation of a TMV-induced HR. Caspase-like protease activity is induced during HR and caspase-1 and -3 inhibitors can prevent TMV-induced HR (del Pozo and Lam, 1998; Chichkova et al. 2004). Both H_2O_2 and NO induced cell death is inhibited by the caspase-1 inhibitor Ac-YVAD-CMK, and therefore, ROI and NO signaling may converge before caspase-1 signaling pathway (Clarke et al. 2000).

Recently, a vacuolar protease (VPE) was shown to have caspase-1 like activity and may trigger HR by aiding in vacuolar collapse (Hatsugai et al. 2004). Silencing of *VPE* suppresses the TMV-induced HR in *NN* plants and increases the protein abundance level of TMV CP (Hatsugai et al. 2004). Further experimentation will have to be conducted to determine if VPE mutants or silencing of VPE causes loss of resistance to TMV. Interestingly, silencing of *VPE* did not increase the protein abundance level of PR-1 and

PR-2. This suggests that VPE belongs to a signaling pathway that regulates HR and possibly *N*-mediated resistance to TMV, but is not necessary for the induction of downstream PR proteins.

Concluding Remarks

In the past decade the inventory of components used during viral recognition and signaling has grown dramatically. The next challenge is to determine how these components form the machinery that drives recognition and signal transduction during disease resistance. Many antiviral *R* genes such as *N, Tm-2*^{2}, *Rx1*, *Rx2*, *Sw-5*, *HRT*, and *RCY1* have been cloned and many of the corresponding viral products that these *R* genes recognize have been discovered. This is an exciting time to study disease resistance signaling to viruses because despite major advances the mechanism for recognition and resistance to viruses is still unclear.

Disease resistance to any pathogen requires a dramatic reprogramming of the cell. Small signaling molecules such as ROIs, SA, JA, ET, and polyamines induce multiple signaling pathways. Many of these pathways function in parallel to one another, while others crosstalk or converge. Consequently, we are faced with a complex signal transduction network whose dissection will require various experimental approaches. For example, powerful genetic approaches have discovered upstream divergence points, such as Rar1, EDS1, and NDR1, as well as downstream convergence points, such as NPR1. Functional genomics have elucidated the interplay between pathways and have implicated gene families, such as the WRKY family of transcription factors. Reverse genetic approaches like VIGS (Burch-Smith et al. 2004) have determined biological roles of JA, ET, and various transcription factors during *N*-mediated resistance to TMV. Thus, as we trek forward, we must carry along these robust, successful approaches and add new tools to our repertoire, such as advanced proteomics and computational biology.

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