Role of Salicylic Acid and NIM1/NPR1 in Race-Specific Resistance in Arabidopsis

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ABSTRACT

Salicylic acid (SA) and the NIM1/NPR1 protein have both been demonstrated to be required for systemic acquired resistance (SAR) and implicated in expression of race-specific resistance. In this work, we analyzed the role that each of these molecules play in the resistance response triggered by members of two subclasses of resistance (*R*) genes, members of which recognize unrelated pathogens. We tested the ability of TIR and coiled-coil-class (also known as leucine-zipper-class) *R* genes to confer resistance to *Pseudomonas syringae* pv. *tomato* or *Peronospora parasitica* in SA-depleted (NahG) and *nim1/npr1* plants. We found that all of the *P. syringae* pv. *tomato*-specific *R* genes tested were dependent upon SA accumulation, while none showed strong dependence upon NIM1/NPR1 activity. A similar SA dependence was observed for the *P. parasitica* TIR and CC-class *R* genes *RPP5* and *RPP8*, respectively. However, the *P. parasitica*-specific *R* genes differed in their requirement for NIM1/NPR1, with just *RPP5* depending upon NIM1/NPR1 activity for effectiveness. These data are consistent with the hypothesis that at least in Arabidopsis, SA accumulation is necessary for the majority of *R*-gene-triggered resistance, while the role of NIM1/NPR in race-specific resistance is limited to resistance to *P. parasitica* mediated by TIR-class *R* genes.

IN angiosperms, resistance (*R*) genes confer race-specific or gene-for-gene resistance to a wide variety of pathogens. Plants containing a specific *R* gene are able to recognize pathogens that carry a corresponding avirulence (*avr*) gene, leading to the activation in the plant of a set of rapid defensive measures at the site of infection, which usually culminate in the generation of reactive oxygen species and localized cell death called the hypersensitive response (HR). Many *R* genes have been cloned from various plants and found to encode proteins that fall into a number of different classes (reviewed in Dangl and Jones 2001). The largest class includes proteins that have a predicted nucleotide binding site (NBS), which is thought to be important for downstream signaling (Bent 1996), and leucine-rich repeats (LRRs), which have been shown to be important for *avr*-protein recognition specificity (Ellis *et al.* 1999; Dodds *et al.* 2001). NBS-LRR R-proteins can be divided into two subclasses that are based on the structure of their amino terminus: one subclass contains a coiled-coil (CC)-like domain (also called a leucine zipper domain), while the other contains a "TIR" domain that has homology to Drosophila Toll and human interleukin-1 transmembrane receptors (Whitham *et al.* 1994; Parker *et al.* 1997).

Pathogen-triggered responses are often accompanied by induction of systemic defense responses that are active against a broad range of pathogens, including viruses, bacteria, and fungi. The best characterized of these is systemic acquired resistance (SAR), which is associated with accumulation of salicylic acid (SA) and a number of pathogenesis-related (PR) gene products (Ryals *et al.* 1996). Many physiological and genetic requirements for both race-specific and SAR have been determined in recent years, and in some cases both processes share these requirements. SAR has been shown to depend upon both SA accumulation and the NIM1/NPR1 protein, which facilitates a systemic response to pathogen-triggered SA accumulation (reviewed by Delaney 1997). A variety of mutants that disrupt *R* gene function have been identified in *Arabidopsis thaliana*. These include mutants that compromise a single *R* gene (*e.g.*, *pbs1*), as well as mutants, such as *eds1*, *ndr1*, *pbs2*, and *pbs3* that show defects in responses to multiple *R* genes (Century *et al.* 1995; Parker *et al.* 1996; Warren *et al.* 1999). Together, these different mutant classes implicate a hierarchical funneling of signals from specific inputs into a few common sets of defense responses. For example, LRR-NBS *R* genes in the TIR or CC class have been shown to require either EDS1 or NDR1, respectively, but not both (Aarts *et al.* 1998). In addition, some *R* genes, such as *RPP7*, *RPP8*, and *RPP13*-Nd, have been shown to act independently of both EDS1 and NDR1, implying the existence of as-yet-undefined *R*-gene signaling pathways (Aarts *et al.* 1998; McDowell *et al.* 2000; Bittner-Eddy and Beynon 2001). Race-specific resistance has also been shown in some, but not all cases to depend upon the SAR

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effectors SA and NIM1/NPR1 (Delaney *et al.* 1994, 1995; Shah *et al.* 1997; Clarke *et al.* 2000; McDowell *et al.* 2000; Feys *et al.* 2001), but no correlation that would predict whether an *R* gene would require SA or NIM1/NPR1 on the basis of its protein structure or pathogen specificity has been established.

We wished to determine whether R-protein structure or pathogen specificity correlated with the requirement for SA accumulation or NIM1/NPR1 function. Therefore, we analyzed the effectiveness of both CC and TIR class *R* genes that recognize *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato* (*Pst*)-produced molecules in NahG and *nim1/npr1* backgrounds. Individual *R* genes within NahG or *nim1/npr1* plants were interrogated by inoculation with various avirulent *Pst* strains or *P. parasitica* isolates, and pathogen growth restriction was compared to that observed on wild-type controls. Our tests included the *Pst*-specific CC *R* genes *RPM1* and *RPS2*, the TIR class gene *RPS4* (Bent *et al.* 1994; Mindrinos *et al.* 1994; Grant *et al.* 1995; Gassmann *et al.* 1999), and *P. parasitica*-specific *RPP5* and *RPP8*, TIR and CC class genes, respectively. *RPP5* and *RPP8* were tested for SA dependence in two independently derived NahG backgrounds, and the effectiveness of these *R* genes in *nim1/npr1* backgrounds was evaluated by testing whether *P. parasitica* resistance segregated with the appropriate *R* gene in *nim1/npr1*-selected F_2 plants derived from crosses between an *R*-gene-carrying accession and *nim1/npr1* mutants in a susceptible accession. All *R* genes tested could be shown to require SA; however, only *RPP5* was shown to require NIM1/NPR1.

MATERIALS AND METHODS

Plants and growth conditions: *A. thaliana* accession Wassilewskija (Ws-0), Columbia (Col-0), and Landsberg *erecta* (L*er*) were obtained from the Ohio State University Arabidopsis Biological Resource Center (Columbus, OH), Ws *nim1-1* and Col NahG plants were described previously in Delaney *et al.* (1994, 1995), and the Ws-NahG line (Molina *et al.* 1998) was provided by Syngenta (Research Triangle Park, NC). *Ler* NahG plants were obtained from Dr. Xinnian Dong (Bowling *et al.* 1994), and *npr1-2* plants were obtained from Dr. Jane Glazebrook (Glazebrook *et al.* 1996). Crosses were performed by emasculating L*er* flowers and applying pollen from the appropriate male parent to the stigma. The success of the crosses was evaluated by testing putative F_1 plants for heterozygosity at the dihydroflavonol 4-reductase (DFR) and g4539 cleaved amplified polymorphic sequence (CAPS) loci. Plants were grown at 22° in short-day conditions (14 hr light, ~150 μE fluence provided by cool white fluorescent lamps) with ~60% relative humidity in Cornell soil mix (Boodley and Sheldrake 1977), composed of 12 ft^3 vermiculite, 7.6 ft^3 peat moss, 4 ft^3 perlite, 5 lb lime, and 4 lb Micromax micronutrient blend (Sierra Chemical, Milpitas, CA).

Molecular genotyping: DNA for CAPS and SSLP analysis was extracted as described in Klimyuk *et al.* (1993). Amplification and cleavage of the PCR products was performed essentially as described (Konieczny and Ausubel 1993; Bell and Ecker 1994). Primers used include DFR, 5′-TGTTACATGGCT TCATACCA-3′, 5′-AGATCCTGAGGTGAGTTTTTC-3′; and CER453919, 5′-ACGGCTTATAGTTGGGCAGTG-3′, 5′-TTT TCGTGGTTTATATCGGGTCAA-3′.

Lactophenol trypan blue staining of *P. parasitica*: To assess *P. parasitica* colonization of inoculated plants, leaves were stained with lactophenol trypan blue and cleared with saturated chloral hydrate, as described (Uknes *et al.* 1993). After the leaves had cleared, chloral hydrate was replaced with 70% glycerol for slide mounting. Whole leaves were analyzed and photographed with a MZ8 stereo microscope (Leica, Wetzler, Germany) and a PM-C 35-mm camera (Olympus, Melville, NY).

Pathogen inoculation and chemical elicitation: *P. parasitica* isolate Noco2 (Crute *et al.* 1993) was provided by Jane Parker (The Sainsbury Laboratory, Norwich, UK) and Emco5 (Holub and Beynon 1997) was provided by Jeff Dangl (University of North Carolina, Chapel Hill, NC). Noco2 and Emco5 were maintained on Col-0 or Ws-0 hosts, respectively, as described in Uknes *et al.* (1992). Inoculum was prepared from plants 8 days postinfection by placing heavily sporulating leaves into water and gently vortexing; the spore suspensions (8×10^4 conidiospores/ml) were misted onto Arabidopsis plants 15 days after sowing, using a compressed air paint sprayer (Preval; Precision Valve, Yonkers, NY), and plants were covered with a clear dome to maintain the high humidity that is optimal for *P. parasitica* germination and growth. Spores to be used in cotyledon assays were pelleted by centrifugation, resuspended in water (8×10^4 conidiospores/ml), and then misted onto plants 5 days after sowing. Chemical induction of SAR was achieved by misting plants with a 0.33 mm suspension of 2,6-dichloroisonicotinic acid (INA; 0.25 mg/ml of a formulation containing 25% INA plus wettable powder), obtained from Syngenta.

***P. syringae* growth measurements:** *P. syringae* pv. *tomato* DC3000 strains were obtained from Dr. Brian Staskawicz (Aarts *et al.* 1998). Inoculation and quantification of *P. syringae* pv. *tomato* DC3000 was performed essentially as described in Tornero and Dangl (2001). Pots containing 2-week-old seedlings were inverted and the plants dipped in a suspension of DC3000 (OD_{600} of 0.05) in 10 mm $MgCl_2$ and 0.02% (v/v) Silwet L-77; seedlings were then placed into a flat that was covered with a plastic dome for 1 hr to maintain humidity, after which the dome was removed. Two plants were then harvested per data point for bacterial quantification; four data points per time point were obtained for each interaction tested. Bacterial quantification was performed as described in Tornero and Dangl (2001).

RNA extraction and analysis: Aerial plant tissue was cut off at the described time points and immediately frozen in liquid nitrogen, and RNA was extracted as in Lagrimini *et al.* (1987). RNA gel-blot analysis was performed as described in Uknes *et al.* (1993). Approximately 5.0 μg total RNA per sample was fractionated by electrophoresis on denaturing 1.2% agarose gels (1× MSE, 3% v/v formaldehyde; Uknes *et al.* 1993). RNA was transferred overnight in 6× SSC to NytranN nylon membranes (Schleicher and Schuell, Dassel, Germany) and then crosslinked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Probes were made using [α-^{32}P]dCTP with a random primer labeling system (GIBCO/BRL, Carlsbad, CA) with Arabidopsis *PR1* and *PR2* cDNA probes (Uknes *et al.* 1992). The PDF1.2 template was amplified by PCR from genomic DNA with the following primers: 5′-CTCATGGCTAAGTTTGCTTCC-3′ and 5′-AATACACACGA TTTAGCACC-3′. Each probe was hybridized to a separate replicate blot containing equally loaded RNA samples. Overnight hybridizations and washes were performed at 65° as described by Church and Gilbert (1984). Radioactivity was detected using a phosphor screen and Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

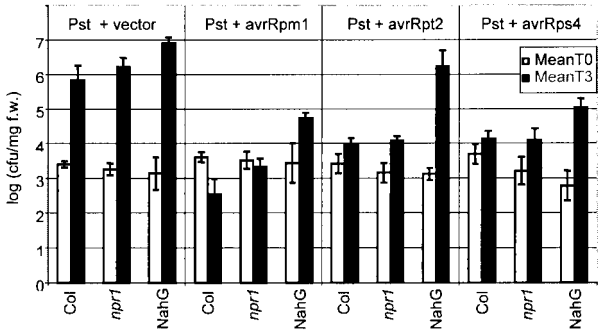

FIGURE 1.—Growth of *P. syringae* pv. *tomato* strains in leaves of *npr1-2* and NahG plants. Wild-type (Col-0), Col *npr1-2*, and Col NahG plants were inoculated by vacuum infiltration with strain DC3000 expressing avrRpm1, avrRpt2, avrRps4, or empty vector alone. Growth of bacteria was assayed immediately following and 3 days after inoculation. Each data point represents the mean ±SE of four samples. The experiment was repeated three times with similar results.

RESULTS

Role of SA and NIM1/NPR1 in race-specific resistance to *P. syringae*: To test whether salicylic acid accumulation or NIM1/NPR1 is essential for transducing signals that originate from different *Pst*-specific *R* genes, we tested their effectiveness in wild-type, *npr1-2*, and salicylate-depleted, NahG-expressing Arabidopsis plants. The *R* genes tested included CC-NBS-LRR-class *RPM1* and *RPS2* (BENT *et al.* 1994; MINDRINOS *et al.* 1994; GRANT *et al.* 1995) and the TIR-class *R* gene *RPS4* (GASSMANN *et al.* 1999). We inoculated accession Col-0, *npr1-2*, and NahG plants with *Pst* DC3000 expressing *avrRpm1*, *avrRpt2*, or *avrRps4*, bacterial avirulence genes that are recognized in the Col-0 accession by RPM1, RPS2, and RPS4, respectively. Growth of the three bacterial strains was significantly greater in the NahG background compared to wild-type plants, demonstrating that SA accumulation plays an important role in the efficacy of each of these *R* genes (Figure 1). However, resistance to all three avirulent DC3000 strains appeared to not be significantly compromised in *npr1-2* plants, indicating that NIM1/NPR1 is not essential to confer robust race-specific resistance to *Pst*.

Role of SA in race-specific resistance to *P. parasitica*: We also analyzed whether SA accumulation was required for the ability of the TIR-class *RPP5* and CC-class *RPP8* *R* genes (PARKER *et al.* 1997; MCDOWELL *et al.* 1998) from L*er* to confer resistance to *P. parasitica*. These tests were conducted by inoculating L*er* NahG plants with *P. parasitica* Noco2 or Emco5, pathogen isolates that are recognized by *RPP5* and *RPP8*, respectively (Table 1A). Hyphal growth was visualized using lactophenol trypan blue staining 10 days after inoculation of L*er* NahG and control L*er* plants. We found that Noco2 was able to colonize L*er* NahG leaves, while Emco5 was not, indicating that *RPP5*, but not *RPP8*, requires SA accumulation for its action (Figure 2A). These results are consistent with those obtained by MCDOWELL *et al.* (2000), who demonstrated that *RPP8*-mediated resistance was expressed in Col NahG plants carrying an *RPP8* transgene, while *RPP4*-mediated resistance to Emoy2, which is likely mediated by an allele of *RPP5* (VAN DER BIEZEN *et al.* 2002), was compromised. These tests with L*er* NahG plants showed that *RPP5*-mediated resistance to Noco2 required SA accumulation, but *RPP8*-mediated resistance against Emco5 did not.

In addition to testing *RPP5* and *RPP8* action in *Ler* NahG plants, we also assessed the SA dependence of the *R* genes in F_1 hybrid plants derived from L*er* × Col NahG or L*er* × Ws NahG crosses. These crosses enabled us to individually interrogate *RPP5* or *RPP8* in the presence of NahG by inoculating the plants with Noco2 or Emco5, respectively (Table 1B). In control crosses between L*er* and wild-type Col-0 or Ws-0 plants, the F_1 plants were resistant to Noco2 or Emco5, respectively, due to action of the dominant heterozygous *RPP5* or *RPP8* loci from *Ler* (Figure 2B). By contrast, L*er* × Col NahG F_1 plants allowed growth of Noco2, confirming

TABLE 1

***P. parasitica* resistance specificities and signaling pathways analyzed in this work**

Genotype inoculated	*P. parasitica* isolate	*R* gene tested	Signal molecule tested	Interaction phenotype[a]
A.				
L*er*	Noco2	*RPP5*		Resistant
L*er* NahG	Noco2	*RPP5*	SA	Susceptible
L*er*	Emco5	*RPP8*		Resistant
L*er* NahG	Emco5	*RPP8*	SA	Resistant
B.				
L*er* × Col-0 F_1	Noco2	*RPP5*		Resistant
L*er* × Col NahG F_1	Noco2	*RPP5*	SA	Susceptible
L*er* × Ws-0 F_1	Emco5	*RPP8*		Resistant
L*er* × Ws NahG F_1	Emco5	*RPP8*	SA	Susceptible
C.				
L*er* × Col-0 F_2	Noco2	*RPP5*		Resistant
L*er* × Col *npr1-2* F_2	Noco2	*RPP5*	NIM1/NPR1	Susceptible
L*er* × Ws-0 F_2	Emco5	*RPP8*		Resistant
Ler × Ws *nim1-1* F_2	Emco5	*RPP8*	NIM1/NPR1	Resistant
D.				
R5N1	Noco2	*RPP5*		Resistant
R5n1	Noco2	*RPP5*	NIM1/NPR1	Susceptible
R8N1	Emco5	*RPP8*		Resistant
R8n1	Emco5	*RPP8*	NIM1/NPR1	Resistant

[a] Genotypes were considered susceptible if they allowed significantly more pathogen growth than the appropriate control.

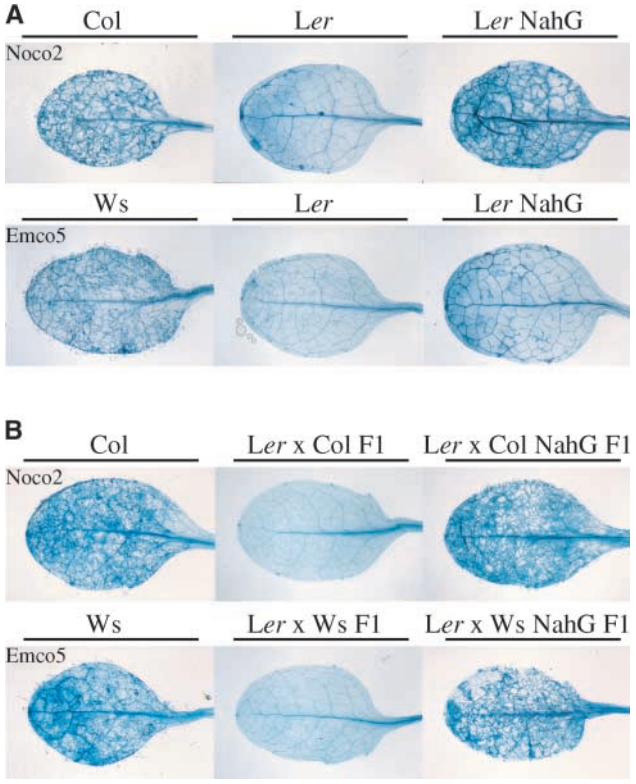

FIGURE 2.—Growth of *P. parasitica* in *nahG*-expressing plants. (A) Plants within a row were inoculated with the *P. parasitica* isolate shown. Compatible wild-type hosts Col and Ws are shown at the left, followed by parental L*er* accessions (middle) and L*er*-expressing NahG (right). (B, center) F_1 plants from L*er* × the susceptible host indicated. (right) F_1 plants from crosses of L*er* and Col NahG or Ws NahG plants. Hyphal growth was assessed by staining with trypan blue 10 days (A) or 8 days (B) after inoculation and comparing growth within a compatible host and control F_1 hybrid not expressing *nahG*. The experiment was repeated three times with similar results.

the observation that *RPP5* action requires SA accumulation. However, unlike L*er* NahG plants, the F_1 hybrids from the L*er* × Ws NahG cross showed extensive hyphal growth and significant sporulation after Emco5 inoculation, indicating that in the hybrids SA does play an important role in RPP8 signaling (Figure 2B). The difference observed between L*er* NahG *vs.* L*er* × Ws NahG hybrids in susceptibility to Emco5 is not simply due to differences in *RPP8* copy number, because *RPP8* heterozygous and homozygous F_2 plants derived from this same cross both fail to express resistance to Emco5 (data not shown). Therefore, in contrast to our observations of L*er* NahG plants, in the hybrid plants, both *RPP5* and *RPP8* required SA accumulation to confer effective resistance to the test pathogens.

Role of NIM1/NPR1 in race-specific resistance to *P. parasitica*: To assess the NIM1/NPR1 dependence of *RPP5*- and *RPP8*-initiated resistance, we analyzed a large number of *nim1/npr1* F_2 plants derived from L*er* × Col *npr1-2* or L*er* × Ws *nim1-1* crosses, in which *RPP5* and *RPP8* would have segregated from their null alleles (Tables 1C, 2, and 3). We chose to perform two separate crosses for these experiments because we found Emco5 growth to be significantly more robust on Ws-0 compared to Col-0, while Noco2 grows only on Col-0. To identify homozygous *npr1-2* or *nim1-1* plants from their respective F_2 population, we applied the *NIM1/NPR1*-dependent, SAR-inducing SA analog INA 3 days before inoculating the population with either Noco2 (for the L*er* × Col *npr1-2* F_2) or Emco5 (for the L*er* × Ws *nim1-1* F_2). Susceptible plants were known to be *nim1/npr1* because of their inability to manifest INA-induced resistance to *P. parasitica* (CAO *et al.* 1994; DELANEY *et al.* 1995), while effective INA-induced resistance was observed in the normally $Nim1^+/Npr1^+$ compatible host controls (Col or Ws) in these experiments (data not shown). Further, susceptible plants must also lack effective *R*-gene action against the test pathogen either due to the absence of the cognate *R* gene or because an *R* gene present failed to function in the *nim1/npr1* background, a determination that was the objective of this experiment. To determine whether *RPP5* or *RPP8* alleles were present in the susceptible *nim1/npr1* plants, sporulating INA-treated plants were genotyped with molecular markers tightly linked to the respective *RPP* genes, and the frequency of the Landsberg allele of that marker was compared to the frequency seen in *P. parasitica*-susceptible L*er* × Col-0 or L*er* × Ws-0 F_2 plants that were not treated with INA. In F_2 plants from these control crosses, susceptible plants would not contain L*er* alleles for molecular markers linked to effective L*er*-derived *R* genes unless a recombination event between the *R* gene and the linked markers had occurred. We analyzed the simple sequence length polymorphism (SSLP; BELL and ECKER 1994) marker CER453919 to genotype the TIR-class *RPP5* locus in Noco2-susceptible

TABLE 2

Cosegregation of resistance with the L*er* CER453919 SSLP in *npr1-2* plants

Population analyzed	R^a	S^b	χ^2	L*er* CER453919/ total alleles in Noco2-susceptible F_2 plants
L*er* × Col F_2	80	30	$P > 0.5^c$	2/60
L*er* × npr1-2 F_2 (INA treated)	128	30	$P < 0.005^d$	17/60

L*er* × Col F_2 and INA-treated L*er* × Col-*npr1-2* F_2 populations were inoculated with Noco2. Noco2-susceptible plants were genotyped with the RPP5-linked SSLP marker CER453919.

[a] Noco2 resistant.

[b] Noco2 susceptible: Plants supporting any conidiophore production were considered susceptible.

[c] χ^2 values are given for the expected ratio of 3:1 (*rpp5*).

[d] χ^2 values are given for the expected ratio of 15:1 (*rpp5 npr1*).

TABLE 3

Cosegregation of resistance with the L*er* DFR CAPS in *nim1-1* plants

Population analyzed	R^a	S^b	χ^2	L*er* DFR/total alleles in Emco5-susceptible F_2 plants
L*er* × Ws F_2	110	28	$P > 0.2^c$	0/56
L*er* × nim1-1 F_2 (INA treated)	310	23	$P > 0.5^d$	0/46

L*er* × Ws F_2 and INA-treated L*er* × Ws-*nim1-1* F_2 populations were inoculated with Emco5. Emco5-susceptible plants were genotyped with the RPP8-linked CAPS marker DFR.

[a] Emco5 resistant.

[b] Emco5 susceptible: Plants supporting any conidiophore production were considered susceptible.

[c] χ^2 values are given for the expected ratio of 3:1 (*rpp8*).

[d] χ^2 values are given for the expected ratio of 15:1 (*rpp8 nim1*).

L*er* × Col and INA-treated L*er* × *npr1-2* F_2 plants (Table 1C). This marker is within 100 kb of RPP5 and should thus be tightly linked to the *RPP5*-mediated resistance phenotype. We found an expected low frequency of L*er* CER453919 alleles (2/60) in Noco2-susceptible L*er* × Col-0 F_2 plants, while a significantly higher frequency of L*er* CER453919 alleles (17/60) was found in Noco2-susceptible plants identified from an INA-treated L*er* × Col *npr1-2* F_2 population (Table 2). These findings indicate that RPP5-mediated resistance is generally compromised in a *nim1/npr1* background and that NIM1/NPR1 therefore does play a significant role in enabling RPP5-mediated resistance. To examine functionality of the CC-class *RPP8* gene in *nim1/npr1* plants, we also assessed the occurrence of the *RPP8*-linked DFR CAPS (Konieczny and Ausubel 1993) marker, which is within 500 kb of the *RPP8* locus. Of 56 alleles examined, we found no L*er* DFR alleles (*i.e.*, all were Ws-0 alleles) from Emco5-susceptible L*er* × Ws F_2 plants, nor did we observe L*er* DFR alleles among 46 chromosomes examined in the Emco5-susceptible L*er* × Ws *nim1-1* F_2 plants, as all 46 carried Ws-0 DFR alleles (Table 3). The similar low frequency of L*er* alleles from the *RPP8*-linked DFR locus in the *nim1-1* and *NIM1* crosses demonstrates that RPP8-mediated resistance functions well in *nim1-1* F_2 plants. Thus, *RPP5* requires a functional NIM1/NPR1 protein to impart resistance, while *RPP8* does not have this requirement.

To confirm the different reliance of these two *RPP* genes on NIM1/NPR1, F_3 plants were obtained from homozygous *RPP5 npr1-2* and homozygous *RPP8 nim1-1* F_2 plants (F_3 lines are henceforth referred to as R5n1 and R8n1, respectively) and tested for their ability to express resistance to Noco2 and Emco5 (Figure 3A). F_3 plants were known to be homozygous *nim1/npr1* mutants, as they failed to express PR-1 3 days after treatment with

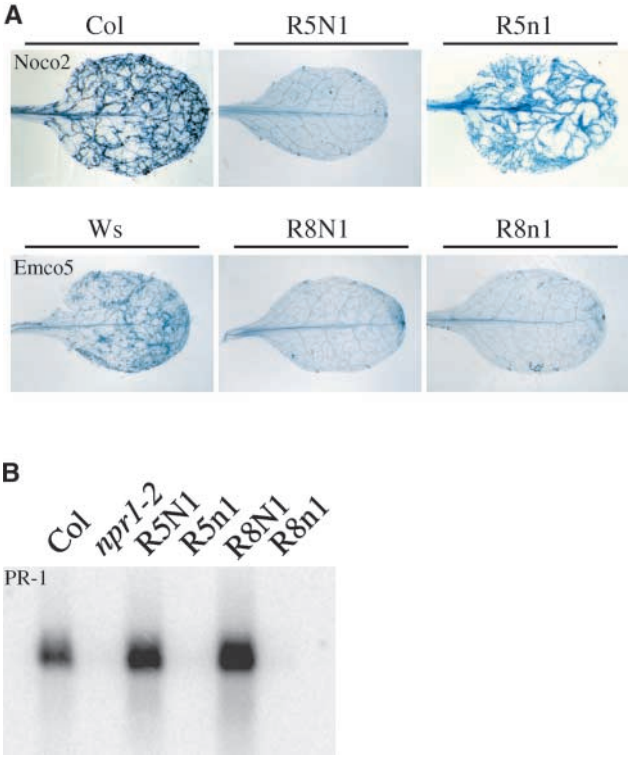

Figure 3.—*RPP* function in *nim1/npr1* plants. (A) F_3 homozygous *RPP5/npr1-2* (R5n1) and *RPP8/nim1-1* (R8n1) plants (right) were inoculated with the indicated *P. parasitica* strain. Parasite structures were stained with trypan blue 8 days after inoculation and compared to hyphal growth in the (left) compatible host and (center) wild-type F_3 plants homozygous for *RPP5/NPR1* (R5N1) or *RPP8/NPR1* (R8N1). (B) RNA gel-blot analysis of *PR1* gene expression 3 days following treatment with INA to confirm the *nim1/npr1* status of these lines. The experiment was repeated three times with similar results.

INA (Figure 3B). For comparison, control *RPP5* and *RPP8* homozygous plants were obtained from corresponding wild-type F_2 populations (R5N1 and R8N1, respectively; both wild type for NIM1/NPR1). Extensive colonization of the R5n1 F_3 plants was seen compared to its wild-type R5N1 counterpart, while no colonization was seen in R8n1 or R8N1 F_3 plants, confirming the results seen in the genetic analysis of the F_2 population.

Quantitative analysis of *RPP* requirements for SA and NPR1/NIM1: To quantitatively assess *RPP* gene requirements for SA and NIM1/NPR1, we measured conidiophore production on cotyledons of young seedlings of various genotypes after inoculation with either Noco2 or Emco5 (Table 4). *RPP5*- and *RPP8*-expressing seedlings that contained or lacked NahG or NIM1/NPR1 function were inoculated 8 days after sowing, and the numbers of conidiophores per cotyledon were scored 8 days later. The results seen in these assays corroborate the findings described above: L*er* NahG plants do not support Emco5 conidiophore production, while L*er* × Ws NahG do, and RPP5-mediated resistance to Noco2 is compromised in both NahG and *npr1-2* backgrounds. R5n1 seedlings

TABLE 4

Disease ratings of Arabidopsis lines following inoculation of 1-week-old seedlings with *P. parasitica* isolate Noco2 or Emco5

	P. parasitica isolate (relevant *R* gene)					
	Noco2 (*RPP5*)			Emco5 (*RPP8*)		
Arabidopsis line[a]	Mean[b]	SEM[c]	*N*[d]	Mean	SEM	*N*
Ws	—	—	—	All > 20[e]	NA	31
Col	All > 20	NA	37	All > 20	NA	30
L*er*	0	0	22	0	0	22
L*er* NahG	3.9	2.7	37	0	0	42
L*er* × Col F_1	0	0	33	0	0	29
L*er* × Col NahG F_1	19.4	6.0	24	0	0	17
R5N1	0.6	1.5	51	—	—	—
R5n1	5.5	2.4	92	—	—	—
L*er* × Ws F_1	—	—	—	5.4	3.3	81
L*er* × Ws NahG F_1	—	—	—	All > 20	NA	42
R8N1	—	—	—	0	0	44
R8n1	—	—	—	0	0	46

NA, not applicable.
[a] R5N1, *RPP5/NPR1*; R5n1, *RPP5/npr1-2*; R8N1, *RPP8/NIM1*; R8n1, *rpp8 nim1-1*.
[b] Number of conidiophores on the most heavily infected cotyledon of each seedling analyzed.
[c] SEM, standard error of the mean.
[d] *N*, number of seedlings analyzed.
[e] All cotyledons were sporulating heavily; number of conidiophores was not determined beyond 20; SEM was not calculated for these populations.

are more susceptible to Noco2 than are R5N1 plants, while R8n1 does not allow Emco5 sporulation.

***RPP5*- and *RPP8*-mediated gene expression:** The observation that RPP5 and RPP8 differ in their requirement for NIM1/NPR1 led us to speculate about whether these two *R* genes induce different sets of defense genes. Therefore, we analyzed the expression of the well-characterized defense genes *PR1*, *PR2*, and *PDF1.2* in L*er* plants inoculated with *P. parasitica* isolate Noco2 or Emco5, which elicited *RPP5*- or *RPP8*-mediated resistance (Figure 4). We were able to see slight differences in the defense-gene expression profiles induced by each pathogen 1 and 2 days after inoculation. In our experience, the higher humidity and lower light intensity of our inoculation environment often leads to nonspecific elicitation of defense gene expression, and we see this response in this experiment most significantly 4 days after treatment. This background expression precluded reliable conclusions regarding gene expression 4 days postinoculation. We noted that 1 and 2 days after inoculation the well-characterized SA and NIM1/NIM1-dependent SAR genes *PR1* and *PR2* were induced much more strongly by RPP5 elicitation than by RPP8, while *PDF1.2* showed greater induction in plants responding to a RPP8 signal. These differences imply that the RPP5- and RPP8-initiated signaling events leading to race-specific resistance initiate distinct downstream transcriptional responses.

DISCUSSION

We demonstrated that all five of the resistance genes tested in this study were compromised by diminished SA levels, regardless of whether they conferred resistance to bacterial or oomycete pathogens and regardless of whether those *R* genes contained a CC or TIR domain. By contrast, *RPP5* is the only *R* gene we were able to show to be significantly compromised by mutations in *NIM1/NPR1*. While this study examined only a subset of known Arabidopsis *R* genes, our observations may form the basis of two broader generalizations: that the majority of Arabidopsis *R* genes require SA accumulation for full resistance activity and that NIM1/NPR1 may play a role only in resistance to *P. parasitica* mediated by TIR-class *R* genes. In support of this hypothesis, the *RPP1* and *RPP4* loci, which confer resistance to Noco2 and Emoy2, respectively, encode TIR-class *R* genes (Parker *et al.* 1997; van der Biezen *et al.* 2002) and resistance mediated by these loci has been shown to be compromised in *nim1/npr1* seedlings (Delaney *et al.* 1995; McDowell *et al.* 2000), although van der Biezen *et al.* (2002) noted that *RPP4* is not significantly compromised in *npr1-1* adult leaves. Also, *RPP13-Nd* is the second CC *P. parasitica R* gene to be cloned and was demonstrated to function independently of *NIM1/NPR1* (Bittner-Eddy and Beynon 2001).

Our data support the hypothesis that functional homology exists for an important defense signal transduc-

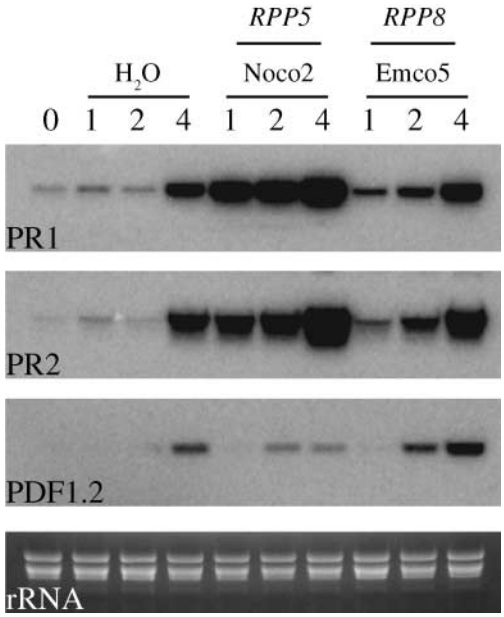

FIGURE 4.—*RPP*-dependent induction of defense genes. Landsberg *erecta* plants were inoculated with 8×10^4 spores/ml of Noco2, Emco5, or mock inoculated with H_2O. At 4 days after inoculation, Noco2- and Emco5-inoculated plants showed no evidence of infection. RNA was isolated from leaves 1, 2, and 4 days after inoculation. RNA gel blots were hybridized with radiolabeled *PR1*, *PR2*, or *PDF1.2* probes as shown. Equal loading of each lane is demonstrated by ethidium bromide staining of rRNA.

tion pathway shared by plants and animals. The predicted *NIM1/NPR1* protein product has similarity to Drosophila Cactus and human IκB proteins (CAO *et al.* 1997; RYALS *et al.* 1997), which transduce signals initiated by the Toll and interleukin-1 receptors, respectively. Interestingly, both Cactus and IκB are important for activation of the innate immune responses in these animals, much like NIM1/NPR1 is required for expression of SAR, a system with many similarities to animal innate immunity. Thus, our finding that an *R* gene containing TIR homology depends upon *NIM1/NPR1* is consistent with the functional conservation of a TIR-like defense pathway in plants, providing evidence for a particularly ancient origin of this signaling pathway that would predate the divergence of plants and animals.

What role does *NIM1/NPR1* play in *RPP5*- and *RPP1*-mediated resistance? While a number of Arabidopsis mutations that seem to directly impair the perception and response to *R* gene elicitation have been isolated, it is unlikely that NIM1/NPR1 plays such a central role in *R* gene signaling. The impairment of *RPP5* by *npr1-2* is less severe than that which results from SA depletion, implying that NIM1/NPR1 plays only a partial role in *RPP5*-initiated responses. It is plausible that, in addition to facilitating SAR, *NIM1/NPR1*-regulated gene induction is rapid enough to play a significant role in limiting the growth of incompatible *P. parasitica*. This is also consistent with the observation that *npr1-2*-compromised *RPP5* resistance was also often associated with trailing necrosis behind the site of hyphal growth (data not shown), suggesting that the HR was elicited, but was too late or insufficient to halt pathogen growth. It is also possible that systemically induced genes regulated by NIM1/NPR1 act synergistically with TIR-class-initiated HR-related responses to prevent *P. parasitica* proliferation.

We should point out that RPP5-mediated resistance is weaker than that initiated by RPP8. While we rarely saw susceptible *RPP5*$^+$ plants in our F_2 populations, the original characterization of this *R* gene noted that it was incompletely dominant (PARKER *et al.* 1993). We did occasionally see a similar phenomenon in F_1 plants: In rare instances, L*er* × Col F_1 plants were found to be slightly more susceptible to Noco2 than were parental L*er* plants (data not shown). In addition, we saw occasional sporulation of Noco2 on the *RPP5 NPR1* F_3 seedlings. By contrast, we never saw sporulation on any RPP8-carrying plant of wild-type background in F_2 or F_3 plants. These observations are noteworthy because *R* genes of a single class may vary in their effectiveness, perhaps owing to the nature of interactions between particular *R*-gene and *avr* gene products. Therefore, it is possible that the differences we observed in NIM1/NPR1 dependence between *RPP5* and *RPP8* may be a consequence of the intensity of the resistance response initiated by those particular *avr-R* gene interactions rather than a qualitative difference in the resistance pathways initiated by *R* genes of differing structure. Further testing of TIR- and CC-class *R* genes will help establish whether R protein structure or its response potency is more predictive of its reliance upon *NIM1/NPR1*.

While a previous study found that RPP8 was functional in NahG-expressing plants (MCDOWELL *et al.* 2000), we demonstrated that, at least in certain genetic backgrounds, SA accumulation is necessary for *RPP8* function. MCDOWELL *et al.* (2000) showed that an $RPP8_{Ler}$ transgene could confer Emco5 resistance in a Col NahG background, and our own experiments demonstrated that L*er* plants expressing the NahG transgene were not compromised in RPP8-specified resistance. However, in L*er* × Ws NahG progeny, we found RPP8 to be impaired in conferring resistance to Emco5. There are a variety of possible explanations for this observation. In RNA gel-blot experiments, we found *nahG* mRNA levels to be significantly higher in the Ws NahG line compared to the Col NahG or L*er* NahG lines used in these studies (our unpublished data), suggesting that the breakdown of RPP8 function in the Ws NahG line may be due to more efficient catabolism of SA in that line compared to the L*er* NahG or Col NahG lines. Alternatively, the discrepancy between the two conflicting conclusions might result from quantitative genetic background ef-

fects that affect *RPP8* efficacy, which may be more evident in an SA-depleted background. This possibility is supported by the observation that cotyledons of L*er* × Ws F_1 seedlings are more susceptible to Emco5 than are L*er* parent seedlings (Table 4), although they are still much less susceptible than L*er* × Ws NahG F_1 seedlings. We do not believe that *RPP8* heterozygosity is necessary to observe SA dependence, as we isolated *RPP8*-homozygous, NahG-expressing plants, which showed comparable levels of Emco5 susceptibility as did the heterozygous plants (data not shown).

If *R* genes of similar structure initiate similar or identical signal transduction pathways, we would expect them to have similar genetic requirements for their function. *HRT* is a closely related *RPP8* paralog (92% amino acid identity), which confers resistance to turnip crinkle virus (Cooley *et al.* 2000). Like *RPP8*, *HRT*-mediated resistance is compromised in a NahG background, but still functional in a *nim1/npr1* background (Kachroo *et al.* 2000). While *HRT* and *RPP8* confer resistance to very different pathogens, it seems likely that they initiate similar responses upon elicitation, given their extensive sequence similarity. The fact that both genes elicit SA-dependent, NPR1-independent resistance supports this hypothesis. Kachroo *et al.* (2000) found that a second locus, *RRT*, regulates HRT-mediated resistance. This locus may also prove to be important to *RPP8*-mediated resistance.

Interestingly, a number of mutants that constitutively exhibit SA-dependent, *NIM1/NPR1*-independent resistance have been identified (Bowling *et al.* 1997; Clarke *et al.* 1998; Clarke *et al.* 2000). Such mutants may shed light on the biochemical events leading to SA-dependent, *NIM1/NPR1*-independent, race-specific resistance, if their resistance phenotype results from inappropriate expression of processes that are normally activated by *R* genes. In support of this idea, analysis of the constitutive *PR* gene expressers *cpr1* and *cpr6* showed that the defense phenotype associated with these two mutations requires *EDS1* (Clarke *et al.* 2001), a gene that is also required to transduce signals that originate from TIR-class *R* genes (Aarts *et al.* 1998).

There is significant evidence that SA plays roles in defense distinct from SAR. Shirasu *et al.* (1997) demonstrated that while exogenous SA does not trigger programmed cell death (PCD) by itself, it is able to potentiate elicitor-triggered PCD at concentrations much lower than those shown to be sufficient to induce SAR. Also, the fungal toxin fumonisin B1 induces PCD in wild-type and *npr1-1* plants but not in NahG-expressing plants (Asai *et al.* 2000). Therefore, it seems plausible that in addition to its sufficiency in inducing SAR at high concentrations, at lower concentrations SA may be a necessary component of the programmed cell death response. SA-dependent, NIM1/NPR1-independent pathways are also important in regulating responses to pathogen elicitation. In a companion study, we described a number of Arabidopsis genes that require SA, but not NIM1/NPR1, for their pathogen-dependent induction (Rairdan *et al.* 2001). In addition, pathogen-elicited accumulation of camelexin, an Arabidopsis phytoalexin, requires SA, but not NIM1/NPR1 (Zhao and Last 1996). By combining the use of pathogens to interrogate individual *R* genes with the growing array of hosts containing defects in defense pathways, the genetic requirements for individual *R*-gene action will be revealed. Important questions persist as to the number of distinct signaling pathways that support race-specific resistance and how these pathways are shared or dedicated for specific pathogen defense responses.

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