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SHORT COMMUNICATION

Downy mildew (*Peronospora parasitica*) resistance genes in Arabidopsis vary in functional requirements for *NDR1*, *EDS1*, *NPR1* and salicylic acid accumulation

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Summary

To better understand the genetic requirements for *R* gene-dependent defense activation in Arabidopsis, we tested the effect of several defense response mutants on resistance specified by eight *RPP* genes (for resistance to *Peronospora parasitica*) expressed in the Col-0 background. In most cases, resistance was not suppressed by a mutation in the SAR regulatory gene *NPR1* or by expression of the *NahG* transgene. Thus, salicylic acid accumulation and *NPR1* function are not necessary for resistance mediated by these *RPP* genes. In addition, resistance conferred by two of these genes, *RPP7* and *RPP8*, was not significantly suppressed by mutations in either *EDS1* or *NDR1*. *RPP7* resistance was also not compromised by mutations in *EIN2*, *JAR1* or *COI1* which affect ethylene or jasmonic acid signaling. Double mutants were therefore tested. *RPP7* and *RPP8* were weakly suppressed in an *eds1-2*/*ndr1-1* background, suggesting that these *RPP* genes operate additively through *EDS1*, *NDR1* and as-yet-undefined signaling components. *RPP7* was not compromised in *coi1*/*npr1* or *coi1*/*NahG* backgrounds. These observations suggest that *RPP7* initiates resistance through a novel signaling pathway that functions independently of salicylic acid accumulation or jasmonic acid response components.

Introduction

The ability of plants to resist pathogen colonization is often dependent upon the expression of naturally variable genes (referred to as *R* genes) conferring race-specific pathogen resistance (Crute and Pink, 1996). Current models suggest that *R* gene-dependent defense responses are triggered by specific interactions between pathogen-encoded ligands and *R* gene-encoded receptors (Keen, 1990). *R* gene action triggers a signal transduction cascade that in turn activates a suite of defense responses such as the rapid death of host cells (hypersensitive response; HR), localized tissue fortification, and antimicrobial gene expression (Hammond-Kosack and Jones, 1996). Many *R* genes have been genetically defined and cloned, and the majority encode proteins with a consensus nucleotide binding site (NBS) and arrays of leucine-rich repeats (LRRs) (Ellis and Jones, 1998). The conservation of these motifs in R proteins that respond to diverse pathogens suggests that R proteins might employ a limited number of signaling pathways.

Mutational screens in Arabidopsis have identified additional components of *R* gene-dependent resistance responses, including several loci that suppress the function of multiple *R* genes and are thus thought to encode signal transduction components (reviewed in Glazebrook *et al.*, 1997). The *ndr1* and *eds1* loci were defined in screens for loss of race-specific resistance to strains of the bacterium

Pseudomonas syringae or the oomycete *Peronospora parasitica* (Century *et al.*, 1995; Parker *et al.*, 1996). *EDS1* and *NDR1* are each required for the function of different subclasses of NBS-LRR *R* genes (Aarts *et al.*, 1998; Century *et al.*, 1995; Parker *et al.*, 1996). In other words, the *R* genes suppressed by the *ndr1* mutation are not affected by *eds1* mutants, and vice versa. This proposed mutual exclusivity in function correlates with R protein structure rather than the types of pathogen recognized by the respective *R* gene products: *eds1* suppresses NBS-LRR resistance proteins with N-terminal motifs similar to the cytoplasmic signaling domain of the Toll and Interleukin1 transmembrane receptors (TIR-NBS-LRR). Conversely, the *ndr1* mutation suppresses NBS-LRR resistance proteins that contain an N-terminal leucine zipper rather than the TIR domain (LZ-NBS-LRR). These observations suggest a model in which *EDS1* and *NDR1* mediate distinct *R* gene-dependent signaling pathways (Aarts *et al.*, 1998).

One probable exception to this apparent rule was noted by Aarts and co-workers. The *RPP8* gene, which encodes an LZ-NBS-LRR protein (McDowell *et al.*, 1998), is not suppressed by either *ndr1* or *eds1* single mutants, as predicted by the model. The *eds1*/*ndr1* double mutant was not tested by Aarts *et al.* so the possibility that *RPP8* signals defense additively through *EDS1* and *NDR1*-dependent pathways was not ruled out. Alternatively, *RPP8*-mediated recognition could be transduced by signaling mechanisms operating independently of *EDS1* or *NDR1*.

Salicylic acid (SA) is a key defense response component in Arabidopsis (reviewed in Durner *et al.*, 1997). Transgenic plants expressing a bacterial salicylate hydroxylase protein that converts SA to catechol (encoded by the *NahG* gene) are compromised in systemic acquired resistance (SAR) (reviewed in Ryals *et al.*, 1996). SA application rescues *eds1* and *ndr1* mutants, suggesting that these components act upstream or independently of SA (Century *et al.*, 1995; Parker *et al.*, 1996). A second key SAR component was identified by mutations in the *NPR*/*NIM1* gene (Cao *et al.*, 1994; Delaney *et al.*, 1995). *NPR1*/*NIM1* operates downstream of SA and encodes a probable transcription regulator (Cao *et al.*, 1997; Ryals *et al.*, 1997). In addition, *npr1*/*nim1* mutants and *NahG* suppress *R* genes that recognize specific isolates of the downy mildew pathogen *P. parasitica* or the bacterium *P. syringae* (Cao *et al.*, 1994; Delaney *et al.*, 1994; Delaney *et al.*, 1995). It has thus been proposed that SA-dependent regulatory components play a central role in *R* gene mediated 'local' resistance as well as SAR (Delaney *et al.*, 1994). However, the effect of *NahG* and *npr1*/*nim1* on numerous *R* genes has not been tested, thus it is not known if SA accumulation and *NPR1* function are universally required for *R* gene dependent defense induction.

Recent studies have revealed SA-independent resistance mechanisms in Arabidopsis, mediated by components of the jasmonic acid (JA) and ethylene (ET) response pathways (reviewed in Dong, 1998). For example, resistance to isolates of the necrotrophic fungi *Alternaria brassisicola* and *Botrytis cinerea* is compromised by a JA response mutant called *coi1* but is unaffected by *NahG* or *npr1*(Thomma *et al.*, 1998). In contrast, the same study revealed that resistance to a *P. parasitica* isolate is unaffected by *coi1*. The jasmonic acid insensitive mutant *jar1* has also been shown to suppress resistance to *Pythium irregulare*, a soilborne oomycete which causes damping-off (Staswick *et al.*, 1998). Thus, SA-dependent resistance mechanisms could be triggered by biotrophs such as *P. parasitica*, while JA-dependent resistance pathways are triggered by necrotrophs. However, the effect of ET and JA response mutations on a large collection of genetically defined, race-specific *R* genes has not been tested.

In this study, we compared the effects of various single and double combinations of disease resistance mutations described above on resistance to eight isolates of *P. parasitica*, in each case conferred by a different *RPP* specificity. We found that *RPP* genes can differ markedly in their dependence upon mutationally defined signal transduction components, and that the *RPP7* gene is not substantially affected by any of the Arabidopsis defense response mutants that have been defined to date.

Results and Discussion

SA accumulation and NPR/NIM *function are not necessary for downy mildew resistance conferred by several* RPP *genes*

We inoculated Colombia (Col-0) lines containing either the *NahG* transgene or the *npr1-1* allele with seven Col-incompatible isolates of *P. parasitica* that are recognized by distinct *RPP* specificities (as described in Experimental procedures). The effects of each mutant on resistance were measured as enhanced hyphal growth and asexual sporulation in mutant backgrounds relative to the wild-type resistance for each isolate (see Experimental procedures; Figure 1). Resistance to Emoy2 and Cand5 appeared to be fully suppressed (heavy sporulation) in Col::*NahG* and partially suppressed (low to moderate sporulation) in Col-*npr1-1* (Figures 1 and 2). In contrast, resistance to Hiks1 was essentially unaffected in Col-*npr1-1* and Col::*NahG*, suggesting that *NPR1* function and SA accumulation are not necessary for *RPP7* resistance. Mutations in the *RPP7* gene conferred full susceptibility to Hiks1, demonstrating that resistance to this isolate in Col-0 is completely dependent upon *RPP7* (Figures 1 and 2, data not shown).

(a)

	Cala2 *RPP2*, Chr.2			Emoy2 *RPP4*, Chr.4			Wela3 *RPP6*, Chr.1		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Col-0	0	0	142	0	0	90	0	0	106
Col-npr1-1	0.2	0.1	66	9.1	0.9	78	0.4	0.1	74
Col::NahG	0.8	0.2	94	19	0.4	88	1.2	0.2	90

	Cand5 not designated			Hind4 not designated			Wand1 not designated		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Col-0	0	0	94	0	0	96	0	0	106
Col-npr1-1	2.7	0.4	62	1.6	0.3	58	0.9	0.1	74
Col::NahG	17.2	0.7	76	0.8	0.1	66	1.4	0.3	70

(b)

	Hiks1 *RPP7*, Chr.1			Emco5 *RPP8*, Chr.5		
	Mean	SEM	N	Mean	SEM	N
Col-0	0.0	0.0	104	16.8	0.6	73
Col-npr1-1	0.0	0.0	45			
Col::NahG	0.0	0.0	48			
Col-rpp7	17.4	0.5	92			
Ler-0				0.0	0.0	74
Col-ndr1-1	0.3	0.1	111	17.3	0.4	43
Ler-eds1-2	0.0	0.0	80	0.4	0.1	44
eds1-2/ndr1-1/RPP8	6.2	0.6	98	2.5	0.3	91
Col-ein2	0.0	0.0	73			
coi1-2	0.1	0.0	135			
coi1-2/npr1-1	0.0	0.0	120			
coi1-2/NahG	0.0	0.0	154			
Col::RPP8				0.0	0.0	100
Col-npr1-1::RPP8				0.1	0.0	101
Col-ndr1-1::RPP8				0.0	0.0	84
Col::RPP8/NahG				1.4	0.4	80
Ws-0				18.6	0.5	21
Ws-eds1-1				19.5	0.2	55
Ws::RPP8				0.6	0.1	90
RPP8/eds1-1				0.2	0.1	103

Figure 1. Asexual reproduction in wild-type and various mutant lines by *P. parasitica* isolates that are recognized by different *RPP* specificities.
Quantitative disease ratings are expressed as the mean number of sporangiophores per cotyledon. SE refers to standard error and N refers to the total number of cotyledons scored in two to three replicates. *RPP8* is segregating 3:1 in the lines derived from the cross to Col::*NahG* lines, and the medium or heavily sporulating lines were omitted from the calculation of mean sporangiophores/cotyledon.

We also assessed the effect of *NahG* and *npr1-1* on the *RPP8* gene, which specifies resistance to the Emco5 isolate of Peronospora. The *RPP8* allele in Col-0 is non-functional, therefore we tested the transgenic Col-0 plants expressing the RPP8 allele from Landsberg *erecta* (*RPP8-Ler*). Resistance to Emco5 in Col-0::*RPP8* was unaffected by *npr1-1* and only slightly suppressed by *NahG*. We observed only rare sporangiophore production and very limited hyphal growth in cotyledons of the latter (Figures 1 and 2). Similarly, *npr1-1* and *NahG* had very weak effects on *RPP* functions specifying resistance to the remaining isolates, Cala2, Wela3, Hind4 and Wand1. Pronounced wilting was observed for Wand1 and Hind4 in Col::*NahG*. This was associated with extensive colonization of tissue with hyphae in the absence of enhanced parasite reproduction.

Two previous papers reported that resistance to Wela was suppressed in Col::*NahG* and Col-*npr1-1* (Delaney *et al.*, 1994; Delaney *et al.*, 1995). The pathogen isolate used in our study, designated Wela3, was derived from a single sexual spore of the original mass Wela spore culture. Thus, the discrepancy in mutant phenotypes we report here may reflect segregation of avirulence determinants between these isolates.

Previous reports demonstrated partial or complete suppression of Ws-*RPP1*, Ws-*RPP12* and *RPS2* (resistance to *P. syringae* expressing *avrRpt2*) in *NahG*, and/or *npr1*/*nim1* backgrounds (Cao *et al.*, 1994; Delaney *et al.*, 1994; Delaney *et al.*, 1995). Furthermore, application of SA or SA analogs, as well as over-expression of *NPR1*, is sufficient to prime resistance to *P. parasitica* (Cao *et al.*, 1998; Uknes *et al.*, 1992). In contrast, our experiments demonstrate that SA accumulation and *NPR1* function are not required for resistance specified by several *RPP* genes. This raises the question of whether SA- and *NPR1*-independent *RPP* genes, such as *RPP7*, activate a completely different resistance mechanism with distinct signaling components and downstream effectors, or whether they activate the same downstream effectors utilized by other *RPP* genes through an alternate signal transduction pathway that bypasses SA and *NPR1*. We intend to utilize DNA microarray analysis, further mutational analysis, and other approaches to test this question.

RPP7 *and* RPP8 *are unaffected by* eds1 *or* ndr1 *mutation, and are only weakly suppressed by an* eds1*/ndr1 double mutant*

RPP8 function (in accession Landsberg erecta (Ler) was not strongly suppressed by either *eds1-2* or *ndr1-1* mutation, although a very slight shift towards susceptibility was observed (Aarts *et al.*, 1998). We observed previously that *rpp8* loss of function alleles in Ler conferred only partial susceptibility to Emco5, even though at least one of the mutations (Ler-*rpp8-4*) causes a severe translational truncation that is probably a null allele (McDowell *et al.*, 1998). Thus, a second, weak *R* gene linked to *RPP8* in Ler could contribute to resistance against Emco5, and may have clouded the interpretations of Aarts and co-workers. We tested the effect of *ndr1* and *eds1* mutations on *RPP8* function (expressed as a transgene) in backgrounds (Col-0 and Wassilewskija (Ws) (Ws-0)) that are fully susceptible to Emco5 infection. *RPP8* in either of these accessions provides strong resistance to Emco5 (Figure 1 and McDowell *et al.*, 1998). We constructed an *ndr1-1*/*RPP8* line in the Col background. An *eds1* allele in Col has not been isolated, therefore we combined the Ws-*eds1-1* allele (Parker *et al.*, 1996) with a Ws::*RPP8* transgene (Experimental procedures). We observed that neither *ndr1-1* nor *eds1-1* had a significant effect on *RPP8* transgene-dependent resistance (Figure 1) other than a very slight increase in hyphal growth. This observation extends the validity of previous observations that the cloned *RPP8* functions independently of *NDR1* and *EDS1*.

Resistance to Hiks1 conferred by *RPP7* is not compromised by *ndr1* (Century *et al.*, 1995), but the effect of *eds1*

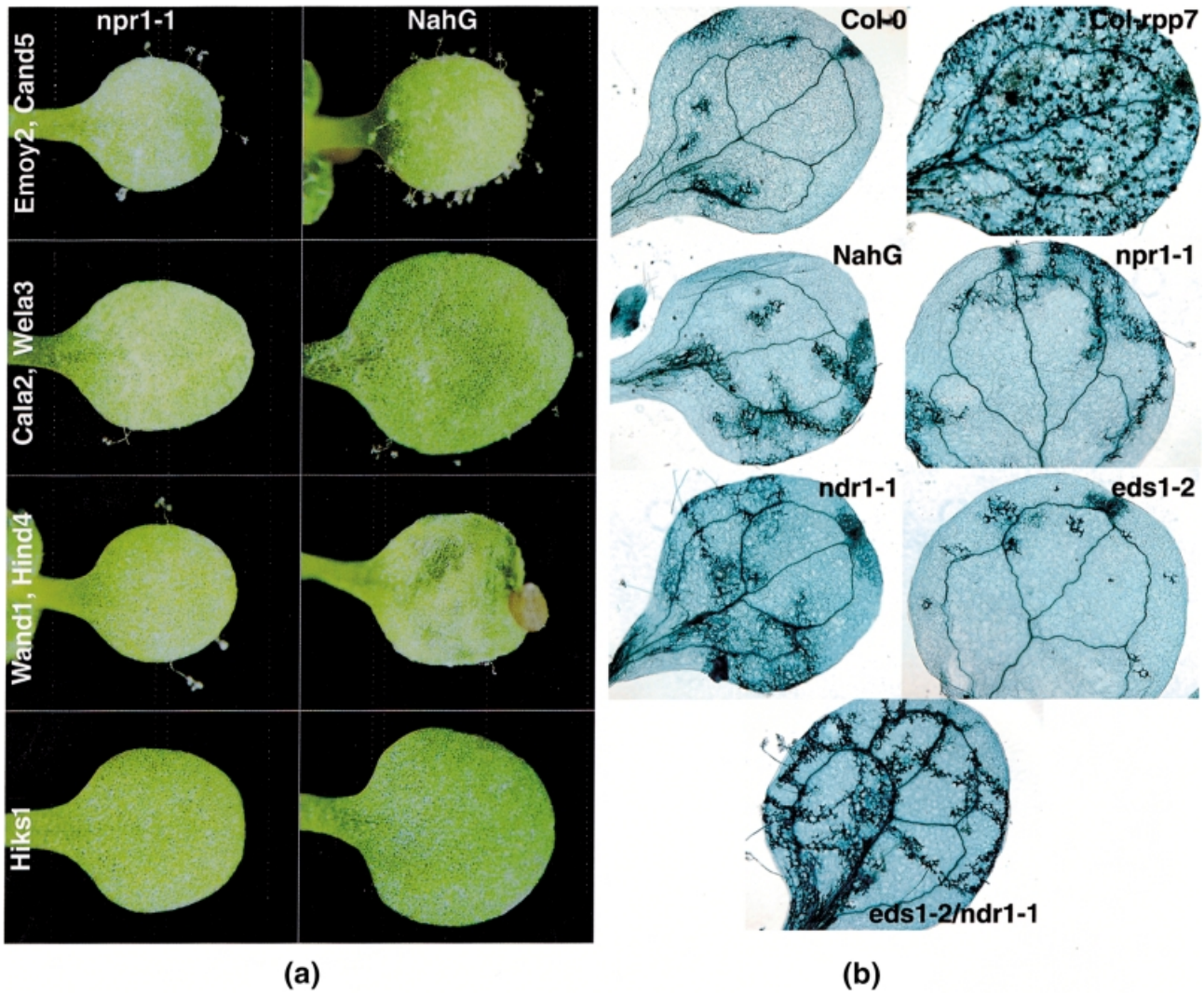

Figure 2. Interaction phenotypes of *P. parasitica* isolates with wild-type Col and Col::*NahG* or Col-*npr1-1*.
(a) Each isolate is recognized by a different *RPP* gene. Cotyledons were photographed 7 days after inoculation.
(b) Interaction of the Hiks1 isolate with various defense response mutants. Cotyledons were stained 7 days after inoculation with Trypan Blue. Col-0 and eds1-2 exhibit hypersensitive cell death at infection sites with no hyphal growth, while Col-*rpp7* supports profuse hyphal growth and oospore production. *ndr1-1*, *NahG*, *npr1-1*, *eds*/*ndr* exhibit patches of trailing necrosis indicative of a delayed resistance response.

mutation on *RPP7* resistance has not been reported. We therefore tested the *eds1-2* allele from Ler. Wild-type Ler is resistant to Hiks1 due to an *RPP* specificity that is either allelic with or closely linked to *RPP7*, based on the observation that no susceptible individuals were found in 2000 F2s from a Col-0 X Ler cross inoculated with Hiks1 (E.B. Holub, unpublished results). We inoculated Ler-*eds1-2* seedlings and found no suppression of Hiks1 resistance in this background. Thus, *RPP7* and *RPP8* are distinct from other known Arabidopsis *R* genes in that they confer resistance independently of both *NDR1* and *EDS1*.

To test whether *eds1-2* and *ndr1-1* have additive effects on *RPP7* or *RPP8* function, we bred a line homozygous for *ndr1-1*, *eds1-2* and *RPP8* (Experimental procedures). Inoculations of this line with Hiks1 and Emco5 revealed only a weak loss of resistance: moderate hyphal growth occurred in infected cotyledons, and light asexual sporulation was observed (Figures 1 and 2). Thus, the combination of *ndr1-1*/*eds1-2* has only a partial effect on *RPP7* or *RPP8* function. Our data, however, demonstrate for the first time that *NDR1* and *EDS1* can act in concert to at least partially transduce *R* function. Thus, the model of a simple dichotomy of signaling pathways subsequent to pathogen recognition based on R protein structure requires modification.

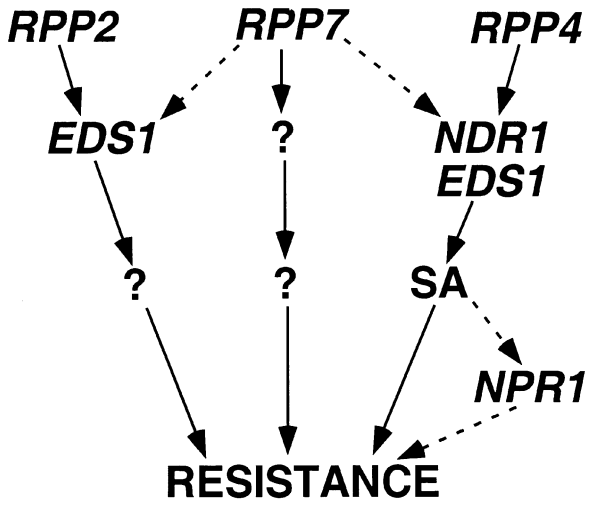

Figure 3. Model for *RPP*-dependent resistance regulation in Arabidopsis, inferred from the dependence or independence of *RPP2*, *RPP4* and *RPP7* on *NDR1*, *EDS1*, *NPR1* and SA accumulation.
Branched pathways represented by dashed lines make relatively weak contributions to the expression of resistance.

RPP7 *is not affected by mutations in the ethylene or jasmonic acid response pathways*

SA and JA signaling can be antagonistic (reviewed in Dong, 1998). The SA-independence of *RPP7* therefore raised the possibility that it operates through a JA- and/or ET-dependent response pathway. We inoculated the Col-*coi1-2* (A. Kloek and B. Kunkel, unpublished results), Col-*ein2.1* and Col-*jar1* (Staswick *et al.*, 1998) mutant lines with Hiks1. Each mutant exhibited wild-type *RPP7* function. Thus, we conclude that *RPP7* resistance is not dependent solely on the ET or JA response pathways. Double mutant combinations of *coi1-2* with either Col::*NahG* or *npr1-1* were also tested and they exhibited wild-type *RPP7* function (Figure 1). This indicates that the SA, JA and ET-independence of *RPP7* resistance cannot be explained by redundant utilization of SA-dependent and JA-dependent response pathways. We thus postulate the existence of a 'third' pathway whose components have not been genetically identified (Figure 3).

It should be noted that *coi1* is a male-sterile line that can only be propagated as a heterozygote, thus all *coi1*-containing lines were segregating for the *coi1* mutation. In each experiment we tested a large enough number of individuals (over 100) to have easily detected susceptible individuals in a segregating population.

Conclusions

The data presented here, in combination with previous studies, suggest the existence of at least three mechanisms by which *RPP* gene-dependent pathogen recognition can be transduced into resistance responses. The first, exemplified by *RPP2*, requires *EDS1* but not SA accumulation or *NPR1*. The second mechanism, exemplified by *RPP4*, requires *EDS1* and SA accumulation and at least in cotyledons is partially dependent upon *NDR1* and *NPR1*. The third mechanism, exemplified by *RPP7* and probably *RPP8*, is partially dependent on the additive functions of *NDR1* and *EDS1* but is independent of SA accumulation and *NPR1*. Furthermore, previous studies demonstrated that *RPP7* is not suppressed by other defense response mutations such as *pbs1*, *pbs2*, *pbs3*, *pad1*, *pad2*, *pad3*, *pad4* or *pad5* (Glazebrook *et al.*, 1997a; 1997b; Warren *et al.*, 1999). We propose that *RPP7* gene function is mediated by a novel regulatory network, either solely or in addition to previously described defense responses. We are conducting large-scale mutational screens to identify additional components of *RPP7*- and *RPP8*-dependent resistance, and have identified at least three new loci that suppress *RPP7* function (J.M. McDowell *et al.*, unpublished results).

Experimental procedures

P. parasitica *isolates*

Seven isolates, each diagnostic for a different *RPP* specificity in Col-0, were used. Wild-type resistance in Col to each isolate is associated with rapid death of penetrated host cells (hypersensitive response), but varies in the restriction of parasite reproduction: three isolates (Cand5, Emoy2 and Hind4) produce low level asexual sporulation (mean 2–4 sporangiophores per cotyledon); one isolate (Cala2) produces a rare sporangiophore in less than 1% of seedlings; and the others (Hiks1, Wand1 and Wela3) never sporulate (Figure 1). Single *RPP* loci have been defined for resistance to Cala2 and Emoy2 (*RPP2* and *RPP4*, respectively, on chromosome 4), and to Hiks1 and Wela3 (*RPP7* and *RPP6*, respectively, on chromosome 1). Resistance to Cand5 and Hind4 appears to be digenic at distinct, independent loci for each isolate (C. Can and E. Holub, unpublished results); locus names have not been designated. Resistance to Wand1 is probably a single locus but unmapped.

Pathogenicity tests

P. parasitica isolates were maintained by weekly subculturing on susceptible recipient plants as described previously (Dangl *et al.*, 1992). Pathogen challenge inoculations were conducted by spraying 7-day-old seedlings with a spore suspension (5×10^4 spores ml^{-1} in dH_2O). Seedlings were grown for 7 days at 16–18°, 8 h day length, 80–100% relative humidity. Asexual sporulation was visually assessed at 7 or 8 days after inoculation by counting sporangiophores on both sides of the cotyledon and classifying individual cotyledons as either N (no sporangia), L (1–10 sporangia), M (11–19) or H (20 or more). We used real numbers (0–10) for N and L cotyledons and assigned values of 15 (M) and 20 (H) to calculate the averages shown in Figure 1. Plants were stained with lactophenol-trypan blue (Koch and Slusarenko, 1990) by boiling for 3 min and continuing the incubation at room temperature overnight. Plants were then de-stained overnight in chloral hydrate and mounted in 70% glycerol for light microscopy.

Construction of RPP8 *transgenic lines, crosses and mutant selection*

One transgenic Col-0 line containing the *pRPP8* plasmid construct (McDowell *et al.*, 1998) was used for crosses with Col-*npr1-1* (Cao *et al.*, 1997), Col-*ndr1-1* (Century *et al.,* 1997), and Col::*NahG* (Weyman *et al.*, 1995). A transgenic Ws-0 line containing *RPP8* in the 9L9 cosmid construct (McDowell *et al.*, 1998) was used for crosses with *Ws-eds1-1* (Falk *et al.,* 1999). In each case the transgenic line was shown by segregation analysis to contain a single transgene insertion locus. The *eds1-2*/*ndr1-1*/*RPP8* triple homozygous line was selected from a Col-*ndr1-1* X Ler-*eds1-2* (Falk *et al.*, 1999) cross. Lines homozygous for Col-*npr1-1*, Col-*ndr1-1*, Ler-*eds1-2* and Ws-*eds1-1* were selected in the F2, F3 or F4 generation with PCR-based markers that distinguished between wild type and mutant alleles. Details of these markers are available upon request. We selected lines that were homozygous for the *RPP8*::*L*er or Col::*NahG* transgene by progeny testing in the F3 or F4 generation for resistance to Basta or Kanamycin. We were unable to isolate F3 lines that were homozygous for both the *RPP8* transgene and the Col::*NahG* transgene, and concluded that these transgenes must reside at linked loci. We were able to isolate a F4 family in which all tested individuals contained the Col::*NahG* transgene. This family segregated 3 : 1 for *RPP8*, and was used in the experiments described above.

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Note added in proof

Klessig and colleagues report that an *RPP8*-like resistance gene against Turnip Crinkle Virus called *HRT* requires SA, but not *NPR1*, ethylene or JA for its function. Mutations in *eds1* and *ndr1* have not been tested for their impact on *HRT* function. Nonetheless, members of the *RPP8* family can differ in their genetic requirements for function (Kachroo *et al.*, 2000. Resistance to turnip crinkle virus in Arabidopsis is regulated by two host genes and is Salicylic Acid dependent but *NPR1*, ethylene and jasmonate independent. *Plant Cell*, in press).