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Research Note

The Arabidopsis Downy Mildew Resistance Gene, *RPP13-Nd*, Functions Independently of *NDR1* and *EDS1* and Does Not Require the Accumulation of Salicylic Acid

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***RPP13-Nd*-mediated resistance prevents parasitism by five isolates of *Peronospora parasitica* (*At*) in a transgenic Arabidopsis. Columbia background. We tested the effect of a number of known disease resistance mutations on the *RPP13-Nd* function and found that resistance remained unaltered in plants carrying mutations in either *EDS1* or *NDR1* and in double *ndr1-1*/*eds1-2* mutant lines. Furthermore, we found that *pbs2*, *pad4-1*, *npr1-1*, and *rps5-1*, which compromise resistance to a number of *P. parasitica (At)* isolates, had no affect on *RPP13-Nd* function. In addition, *RPP13-Nd*-mediated resistance remained unchanged in a background of salicylic acid depletion (*nahG*). We conclude that RPP13-Nd is the first Arabidopsis *R* gene product reported to act via a novel signaling pathway that is independent of salicylic acid-mediated responses and is completely independent of NDR1 and EDS1.**

Plants protect themselves from microbial attack by utilizing preformed defenses and the local and systemic induction of a range of physiological, biochemical, and molecular responses that culminates in disease resistance (Hammond-Kosack and Jones 1996). In many plant–pathogen interactions, the presence or absence of a complementary pairing of a host resistance (*R*) gene and pathogen avirulence (*avr*) gene determines whether molecular recognition occurs and subsequent disease resistance responses are triggered, i.e., "gene-for-gene resistance" (Baker et al. 1997; Flor 1971). *Arabidopsis thaliana R* genes conferring resistance to necrogenic *Pseudomonas syringae* bacteria (Bent et al. 1994; Gassmann et al. 1999; Grant et al. 1995; Mindrinos et al. 1994; Warren et al. 1998) and the obligate biotrophic oomycete *Peronospora parasitica* (*At*) (we are following the nomenclature proposed by Rehmany et al. (2000) to describe *P. parasitica* isolated from *A. thaliana* hosts) have been cloned (Bittner-Eddy et al. 2000; Botella et al. 1998; McDowell et al. 1998; Parker et al. 1997). Conserved structural motifs found within the encoded proteins indicate that these *R* genes may employ similar disease resistance signaling mechanisms. Each protein has a C-terminal domain of variable length, consisting of leucine-rich repeats (LRRs) coupled to a consensus nucleotide binding site (NBS) and either an N-terminal domain comprising a putative leucine zipper (LZ) or a region with similarity to the cytoplasmic signaling domains of *Toll* and interleukin-1 (the TIR domain).

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Mutational screens in Arabidopsis have revealed additional disease resistance components. The *PAD4* gene encodes a lipase-like protein that acts upstream of salicylic acid (SA) in SA-mediated disease resistance (Jirage et al. 1999; Zhou et al. 1998). *pad4* plants show enhanced susceptibility to virulent *P. syringae* strains and gene-for-gene resistance to a number of avirulent isolates of *P. parasitica* (*At*) is suppressed (Glazebrook et al. 1997). The *NPR1–NIM1*/*SAI1* gene acts downstream of SA as a positive regulator of pathogenesis-related genes *PR1, PR2,* and *PR5* and controls the onset of systemic acquired resistance (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Mutations in the *NPR1*/*NIM1*/*SAI1* gene also affect gene-for-gene resistance to some, but not all, *P. parasitica* (*At*) isolates (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Holub and Beynon 1997; McDowell et al. 2000). SA is required for the expression of local resistance to *P. syringae* and *P. parasitica* (*At*) and to restrict the severity of disease caused by virulent forms of these pathogens and the biotrophic fungal parasite *Erysiphe orontii* (Delaney et al. 1994; Mauch-Mani and Slusarenko 1996; Reuber et al. 1998). McDowell et al. (2000), however, demonstrated that accumulation of SA is not required for the function of a number of *P. parasitica* (*At*) *R* genes.

EDS1 and *NDR1* define two signaling pathways that confer gene-for-gene resistance in Arabidopsis to a nonoverlapping set of *P. syringae* strains and *P. parasitica* (*At*) isolates (Century et al. 1995; Century et al. 1997; Parker et al. 1996). Aarts et al. (1998) found that *NDR1*-dependent *R* genes did not require *EDS1*, whereas those *R* genes that were dependent on *EDS1* expressed a slight reduction in resistance in a *ndr1* mutant background. Of the *R* genes whose structure was known, the *EDS1*-dependent class encoded TIR/NBS/LRR-type proteins and the *NDR1*-dependent class encoded

LZ/NBS/LRR-type proteins. A caveat to this association is that *RPP8-Ler*, which encodes a LZ/NBS/LRR-type protein (McDowell et al. 1998) did not require *NDR1* (Aarts et al. 1998). Interestingly, *RPP8-Ler* and another *P. parasitica* (*At*) *R* gene, *RPP7*, are partially dependent on the additive functions of *NDR1* and *EDS1*. McDowell et al. (2000) constructed double mutant lines and found that a weak but significant reduction in *RPP8-Ler* and *RPP7*-mediated resistance occurred.

We recently cloned *RPP13-Nd* from the Niederzenz (Nd-1) accession of Arabidopsis, an *R* gene that confers resistance to at least five isolates of *P. parasitica* (*At*) (Bittner-Eddy et al. 1999; Bittner-Eddy et al. 2000). The RPP13-Nd protein is structurally related to other LZ/NBS/LRR-type R proteins, yet outside the conserved NBS only shows significant homology to the LZ motif of *RPP8-Ler* and to an LRR element conserved within other LRR domains from *R* genes of this class (Bittner-Eddy et al. 2000; Warren et al. 1998). Given the homology between the LZs of *RPP8-Ler* and *RPP13-Nd*, we tested whether *NDR1* or *EDS1* were required for *RPP13-Nd*-mediated resistance. Most Arabidopsis mutations, including *ndr1-1*, are in the Columbia (Col-0) accession; hence, it was necessary to combine *RPP13-Nd* with the particular Col-0 mutant. We used Col::*RPP13-Nd* transgenic lines containing a single insertion locus for all crosses rather than Nd-1 in order to avoid problems of interpretation as a result of gene segregation in different genetic backgrounds. The *eds1-1* mutation, however, is in the Ws background so the F_2 progeny will have a mixed background in this cross; no mutant Col-0 allele has been characterized. F_1 plants were selected by resistance to the selectable marker D-phospinothricin (PPT) carried by the male Col::*RPP13-Nd* parent, and the resulting F_2 progeny were inoculated with the Emco5 isolate (7-day-old seedlings were sprayed with inoculum at 5×10^4 asexual spores per ml). Emco5 is compatible (virulent) on Col-0 and Ws (Holub and Beynon 1997) but incompatible (avirulent) on Col::*RPP13-Nd* as a result of expression of the *RPP13-Nd* transgene (Bittner-Eddy et al. 2000). Table 1 shows segregation of Emco5 resistance among the F_2 populations and χ^2 values (one degree of freedom) calculated for ratios of 3:1 and 9:7 (resistant to susceptible). For both sets of crosses, the observed ratios approximated 3:1 ($0.5 < P < 0.9$). A 3:1 ratio would be expected if susceptibility was a result of segregation of the dominant *RPP13-Nd* gene alone. This result suggests that neither *NDR1* nor *EDS1* are required for *RPP13-Nd* function. Furthermore, we observed an absolute correlation between Emco5 susceptibility and PPT sensitivity (100 µg per ml), confirming that disease was a result of segregation of *RPP13-Nd* (data not shown). We also found that *ndr1-1* and *eds1-1* did not compromise resistance to the other isolates recognized by *RPP13-Nd* (data not shown). We omitted Maks9 from the analysis with Ws-*eds1-1* because of the complication arising from the Ws gene *RPP1* that also recognizes this isolate (Botella et al. 1998).

Col-0 mutations *pbs2*, *pad4*, *npr1-1*, and *rps5-1* also compromise gene-for-gene resistance to *P. parasitica* (*At*), and we examined their effect on the expression of *RPP13-Nd*-mediated resistance. We used two Col::*RPP13-Nd* transgenic lines, each with a single transgene locus, for crosses to these lines and to Col-*ndr1-1* and Col::*nahG* plants to generate homozygous lines for analysis. F_1 plants were selected by PPT resistance as before and, on the basis of reported observations, the incompatible isolates Emoy2 or Cala2 were used to identify F_2 progeny likely to be homozygous for the particular mutant allele. Segregation of PPT resistance among F_3 seedlings was used to select F_2 parents that were homozygous for the *RPP13-Nd* transgene. We then confirmed that these F_2 parents were homozygous for *pbs2*, *rps5-1*, or *pad4-1* by sequencing the mutant alleles or by specific primers to detect the *ndr1-1* deletion. Plants homozygous for *nahG* were identi-

Table 1. Segregation of resistance among F_2 progeny of four independent Col::*RPP13-Nd* transgenic lines crossed to Col-*ndr1-1* or Ws-*eds1-1* following inoculation with the Emco5 isolate of *Peronospora parasitica* (*At*)

	Interaction phenotypes[a]		Chi square analysis[b]	
Arabidopsis accession–Cross	**R[c]**	**S[d]**	**χ^2 (3:1)**	**χ^2 (9:7)**
Columbia (Col) wild-type	0	18		
Col::*RPP13-Nd* line 1	24	0		
Col-*ndr1-1*	0	23		
Ws wild-type	0	25		
Ws-*eds1-1*	0	19		
Line 1 × Col-*ndr1-1*	180	62	0.022	31.59
Line 2 × Col-*ndr1-1*	171	61	0.144	28.02
Line 9 × Col-*ndr1-1*	188	58	0.195	39.86
Line 10 × Col-*ndr1-1*	140	45	0.016	27.58
Line 1 × Ws-*eds1-1*	137	41	0.270	30.20
Line 2 × Ws-*eds1-1*	138	42	0.185	29.68
Line 9 × Ws-*eds1-1*	153	51	0.000	28.38
Line 10 × Ws-*eds1-1*	159	50	0.075	32.57

[a] Data presented is from a single experiment with the Emco5 isolate. The experiment was repeated twice with similar results. Isolates Aswa1, Edco1, Goco1, and Maks9 gave similar segregation ratios as Emco5 from two independent experiments. Isolate Maks9 was not tested on the Col::*RPP13-Nd* × Ws-*eds1-1* material.

[b] Chi square analysis was performed on the data for two segregation ratios. If *RPP13-Nd* functions independently of either *EDS1* or *NDR1*, then a segregation ratio of 3:1 (resistant–susceptible) would be expected on the basis of the segregation of the dominant *R* gene in the F_2 populations. If either *eds1-1* or *ndr1-1* affected *RPP13-Nd*-mediated resistance, then a segregation ratio of 9:7 would be expected, reflecting segregation of two genes required for resistance.

[c] Seedlings were assessed as resistant (R) if no asexual sporulation was seen 7 days postinoculation.

[d] Seedlings were assessed as susceptible (S) if there was any sign of asexual sporulation. In this experiment this typically meant > 20 sporangiophores per inoculated cotyledon.

fied by screening for lack of segregation of the selective *npt2* resistance marker, and plants homozygous for *npr1-1* were identified by testing F_3 progeny on medium containing 0.5 mM SA (Cao et al. 1997).

We confirmed that *RPP13-Nd*-mediated resistance did not require *NDR1*. We observed no asexual sporulation on Col::*RPP13-Nd*/*ndr1-1* seedlings following Emco5 inoculation (Fig. 1A). Furthermore, we saw no evidence of hyphal growth to indicate even a partial loss of resistance as a result of *ndr1-1*. Typical examples of trypan blue-stained (Koch and Slusarenko 1990) cotyledons from our study are shown in Figure 1B. Trypan blue will stain the hyphae of *P. parasitica* (*At*) (see example of wild-type Col-0 infected with Emco5 in Fig. 1) and plant cells that have undergone a hypersensitive resistance response. As a control, we used Emoy2, which exhibits increased growth on all Col-0 mutants analyzed here and on Col::*nahG* (Beynon and Holub 1997; Glazebrook et al. 1997; Warren et al. 1998; Warren et al. 1999) (Fig. 1C). The *pbs2* mutation affects the same set of *R* genes as *ndr1-1*, although for some interactions suppression of gene-for-gene resistance by *pbs2* is several-fold greater than it is by *ndr1-1* (Warren et al. 1999) (Fig. 1C). We tested the possibility that *pbs2* may also exert a stronger effect on *RPP13-Nd*-mediated resistance than does *ndr1-1*. We saw no change in resistance in our Col::*RPP13-Nd*/*pbs2* seedlings following inoculation with Emco5 or any of the other four isolates (Fig. 1A and B and data not shown). This finding is also consistent with our initial observation that *RPP13-Nd* functions independently of *EDS1* because *pbs2* also weakens resistance specified by *EDS1*-dependent *R* genes in addition to those requiring *NDR1* (Warren et al. 1999).

The *rps5-1* mutant allele has a nonconservative amino acid substitution in an LRR, conserved between some *R* genes, of the *P. syringae* resistance gene *RPS5* (Warren et al. 1998). We noted that the LRR altered in *rps5-1* also was conserved in *RPP13-Nd* (Bittner-Eddy et al. 2000). Resistance specified by several *RPP* and *P. syringae* resistance genes, in addition to *RPS5*, are partially compromised by *rps5-1*. Warren et al. (1998) speculated that this might be a result of the sequestering of a protein required for the function of multiple *R* genes by *rps5-1*. This protein might also be required by RPP13-Nd. We tested this possibility, but observed no change in resistance to Emco5 in our Col::*RPP13-Nd*/*rps5-1* seedlings (Fig. 1A and B), indicating that RPP13-Nd does not require this hypothetical protein or is capable of competing effectively for it against *rps5-1*.

Expression of the bacterial salicylate hydroxylase (*nahG*) gene in transgenic Col plants prevents SA accumulation and renders the plants susceptible to infection by the incompatible *P. parasitica* (*At*) isolates Wela and Emwa (Delaney et al. 1994). We used the Col::*nahG* plant to examine the role of SA in *RPP13-Nd*-mediated resistance. No shift in disease resistance phenotype was observed in any of the seedlings or tissue examined following inoculation with Emco5 (Fig. 1A and B) or with any of the other four isolates recognized by *RPP13-Nd* (data not shown). We believe this effect is not a result of poor

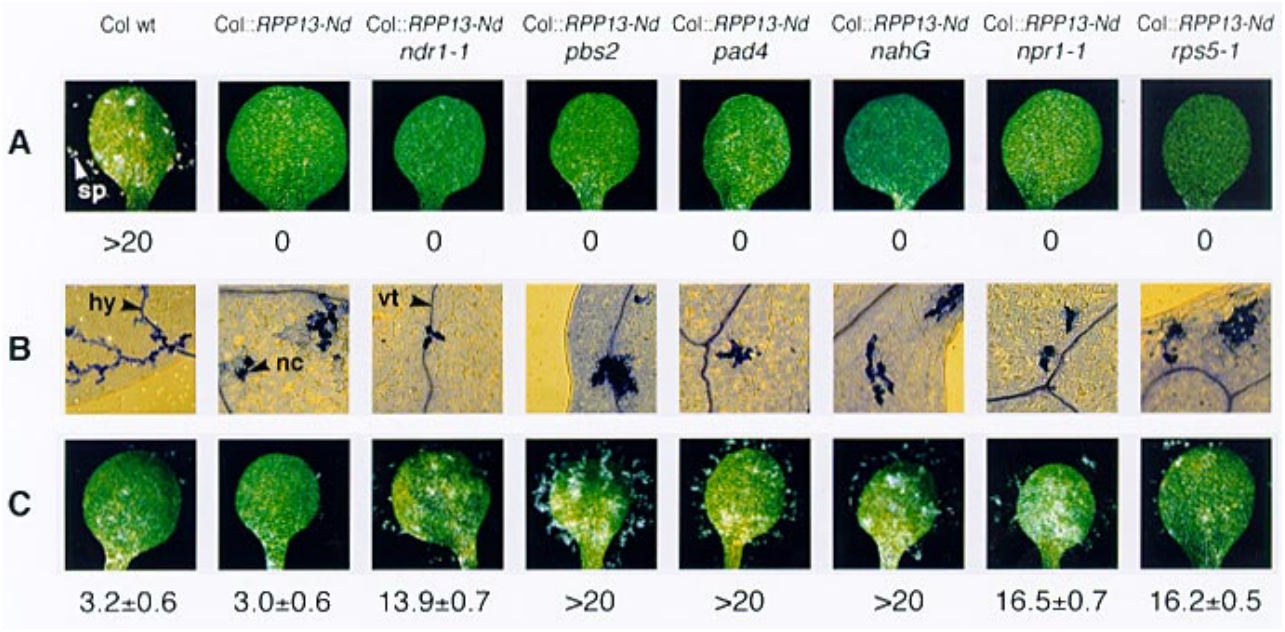

Fig. 1. *RPP13-Nd* function is unaffected by *ndr1-1*, *pbs2*, *pad4*, *npr1-1*, *rps5-1*, and SA depeletion (*nahG).* Photographs of cotyledons or cleared cotyledon tissue were taken 7 days after spray inoculation with Emco5 or Emoy2 (5×10^4 asexual spores per ml). Typical examples are shown. Approximately 100 seedlings distributed across four pots were inoculated for each line tested. Results were consistent across two experiments and for each of the two independent Col::*RPP13-Nd* lines. The amount of asexual sporulation was quantified and expressed as the mean number of sporangiophores (sp) per cotyledon. Seedlings from one pot (20 to 25) were sampled for each line. **A,** *RPP13* from the Nd-1 accession confers resistance to Emco5 in transgenic Col plants and is unaffected by the various disease response mutants. Heavy asexual sporulation (> 20 sporangiophores per cotyl edon) is seen on Col wild type in contrast to the lack of sporulation on the transgenic Col::*RPP13-Nd* line and on any of the homozygous transgene–mutant lines. **B,** The ability of *RPP13-Nd* to restrict the growth of Emco5 is not compromised in the mutant backgrounds. Cotyledons were stained with trypan blue (Koch and Slusarenko 1990) 7 days postinoculation with Emco5, cleared, and viewed at low magnification under a light microscope. Hyphae (hy), necrotic host cells (nc) expressing a hypersensitive resistance response and vascular tissue (vt) are indicated. **C,** *RPP4* function is compromised by *ndr1-1, pbs2, pad4, npr1-1, rps5-1,* and SA depeletion (*nahG).*

nahG expression in our crosses because we observed a shift in the level of asexual sporulation on these plants following inoculation with the incompatible isolate Emoy2 (Fig. 1C). *RPP13-Nd*, therefore, presumably does not require SA-mediated resistance. To test this, we used *pad4* and *npr1-1*, both of which are compromised in SA-mediated signaling (Cao et al. 1994; Cao et al. 1997; Glazebrook et al. 1996; Glazebrook et al. 1997; Jirage et al. 1999; Zhou et al. 1998). The *npr1-1* mutant is suppressed in resistance to some incompatible isolates of *P. parasitica* (*At*), including Emoy2 (Holub and Beynon 1997). In tests with our Col::*RPP13-Nd*/*npr1-1* lines, we observed decreased resistance to Emoy2, as expected (Fig. 1C), but resistance to Emco5 was not compromised (Fig. 1A and B). Furthermore, we found that *RPP13-Nd*-mediated resistance remained unchanged in the *pad4* background (Fig. 1A and B). Together these results strongly suggest that *RPP13-Nd*-mediated resistance does not require SA-dependent resistance responses. In a survey of eight other *RPP* genes, including *RPP7* and *RPP8-Ler*, McDowell et al. (2000) found that most did not require *NPR1* or SA accumulation for function. In contrast to the results reported here for *RPP13-Nd*, however, resistance conferred by these *RPP* genes was slightly suppressed as revealed by an increase in intercellular hyphal growth and, in some instances, light asexual sporulation. Enhanced susceptibility is a feature of plants expressing *nahG* or carrying the *pad4* or *npr1* mutations, indicating that there may be a weak level of resistance inherent in plants expressing a compatible interaction that is SA-dependent. It is interesting then that several SA-dependent *RPP* genes, for example *RPP4*, are not as effective as *RPP13-Nd* in restricting growth of their respective incompatible isolate, often allowing a limited amount of asexual sporulation (Fig. 1C). SA-independent *RPP* gene function may be determined by *R* gene structure and/or the effectiveness with which primary disease resistance responses such as the production of reactive oxygen species (Hammond-Kosack and Jones 1996) are able to inhibit an obligate parasite like *P. parasitica* (*At*). The speed of such primary responses may dictate whether or not secondary responses such as those mediated by SA are required for preventing any pathogen growth beyond the original point of invasion.

RPP13-Nd and *RPP8-Ler* encode LZ/NBS/LRR-type proteins that function independently of *NDR1*. Resistance to *P. syringae* bacteria conferred by LZ/NBS/LRR-type *R* genes *RPS2* (Bent et al. 1994; Mindrinos et al. 1994), *RPS5* (Warren et al. 1998), and *RPM1* (Grant et al. 1995) is abolished in *ndr1-1* plants (Century et al. 1995). The fact that resistance to the Cand5 isolate of *P. parasitica* (*At*) also is abolished in *ndr1-1* plants (Century et al. 1995), however, indicates that *R* gene dependence on *NDR1* is not dictated by pathogen type. Apparent *R* gene independence from *NDR1* may be explained by functional redundancy of the *NDR1* and *EDS1* disease resistance pathways. Recently, McDowell et al. (2000) tested this possibility for two *RPP* genes, *RPP7* and *RPP8-Ler*, by constructing a double mutant line from Col-*ndr1-1* and Ler-*eds1-2*. These authors found a small but significant reduction in *RPP7* and *RPP8-Ler* function in their double mutants, which they did not observe in the single mutant lines. Therefore, *NDR1* and *EDS1* make a contribution to resistance governed by these two genes, although an additional signaling pathway(s) also must exist for full resistance. Given the parallels with *RPP8-Ler*, we examined whether this might also be the case for *RPP13-Nd*. We used a line homozygous for *ndr1-1*, *eds1-2*, and *RPP8-Ler* (McDowell et al. 2000) to cross to one of our Col::*RPP13-Nd/ndr1-1* lines. The resulting F_2 progeny are a mix of the Col-0 and Ler accessions. Molecular markers were used to select a line with the genotype *ndr1-1*/*eds1-2*/*RPP13-Nd* that also lacked *RPP8-Ler*, which was necessary because both *R* genes confer resistance to Emco5 (Bittner-Eddy et al. 2000; McDowell et al. 1998). We inoculated approximately 50 *ndr1-1*/*eds1-2*/*RPP13-Nd*/*rpp8* seedlings in two separate experiments. We observed no asexual sporulation, yet inoculation of *ndr1-1*/*eds1-2*/*rpp13*/*RPP8-Ler* seedlings resulted in a similar level of light asexual sporulation, as reported by McDowell et al. (2000) (data not shown). We examined tissue following trypan blue staining and found that, unlike *RPP8-Ler*, resistance conferred by *RPP13-Nd* in the *ndr1-1*/*eds1-2* double mutant remained unaffected, with no evidence of Emco5 hyphal growth (Fig. 2).

Therefore, *RPP13-Nd* is unique among known Arabidopsis *R* genes in that it is not dependent on either *NDR1* or *EDS1*

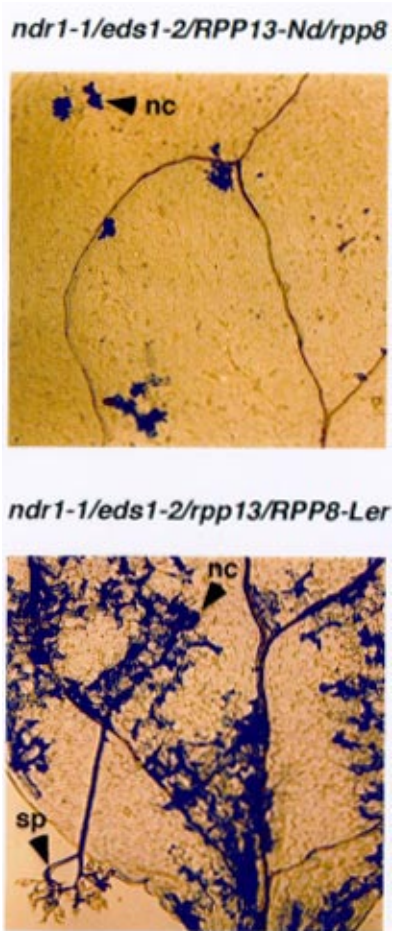

Fig. 2. *RPP13-Nd*-mediated resistance remains unaffected in an *ndr1-1*/*eds1-2* double mutant background. Cotyledons were stained with trypan blue 7 days after spray inoculation with Emco5. Typical examples are shown. Discrete foci of hypersensitive host cells in the *ndr1-1*/*eds1-2*/*RPP13-Nd*/*rpp8* genotype contrasts with the trailing necrosis and presence of sporangiophores (sp) seen in the *ndr1-1*/*eds1-2*/*rpp13*/*RPP8-Ler* genotype as a result of a delayed resistance response. nc = necrotic host cells.

for full function and potentially utilizes a unique disease resistance pathway. We plan to screen mutagenized seed from a Col::*RPP13-Nd* transgenic line to identify components of such a pathway. McDowell et al. (2000) also concluded from their analyses that the function of *RPP7* and *RPP8-Ler* are dependent on novel pathways. Interestingly, *RPP13-Nd* and *RPP8-Ler* confer resistance against the Emco5 isolate, although it is unknown whether the same *avr* gene product is recognized. Mutants identified from our screen will allow us to test whether resistance conferred by these two *R* genes to Emco5 requires the same signaling pathway and determine the effect such mutations have on *RPP7* function.

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