Identification of Arabidopsis Mutants Exhibiting an Altered Hypersensitive Response in Gene-for-Gene Disease Resistance

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Accepted 22 November 1999.

A mutational study was carried out to isolate *Arabidopsis thaliana* plants that exhibit full or partial disruption of the *RPS2*-mediated hypersensitive response (HR) to *Pseudomonas syringae* that express *avrRpt2*. Five classes of mutants were identified including mutations at *RPS2*, *dnd* mutations causing a “defense, no death” loss-of-HR phenotype, a lesion-mimic mutant that also exhibited an HR phenotype, and a number of intermediate or partial-loss-of-HR mutants. Surprisingly, many of these mutants displayed elevated resistance to virulent *P. syringae* and, in some cases, to *Peronospora parasitica*. Constitutively elevated levels of pathogenesis-related (PR) gene expression and salicylic acid were also observed. In the lesion-mimic mutant, appearance of elevated resistance was temporally correlated with appearance of lesions. For one of the intermediate lines, resistance was shown to be dependent on elevated levels of salicylic acid. A new locus was identified and named *IHR1*, after the mutant phenotype of “intermediate HR.” Genetic analysis of the intermediate-HR plant lines was difficult due to uncertainties in distinguishing the partial/intermediate mutant phenotypes from wild type. Despite this difficulty, the intermediate-HR mutants remain of interest because they reveal potential new defense-related loci and because many of these lines exhibit partially elevated disease resistance without dwarfing or other apparent growth defects.

Additional keywords: *dnd1*, *dnd2*.

The cloning of plant disease resistance genes, pathogen avirulence genes, and other defense-associated genes has presented new opportunities to understand the pathogen recognition and defense signal transduction processes that underlie disease resistance. While study of these known genes and proteins is in progress, the identification of additional compo-

nents of plant defense pathways remains a high priority. Mutational strategies have been particularly effective for the identification of genes that influence plant defense. For example, mutants of *Arabidopsis thaliana* have been used by a number of researchers to identify genes involved in plant defense (Dong 1998; Innes 1998).

In earlier screens that investigated the resistance of *Arabidopsis* to *P. syringae* that express *avrRpt2*, seven mutant alleles of *RPS2* were found but no loci other than *RPS2* were identified (Kunkel et al. 1993; Yu et al. 1993). There are many possible explanations for this recurrent identification of *rps2* mutations to the exclusion of other genes in the *avrRpt2-RPS2* response pathway. Lethality of functional disruption could prevent identification of other relevant mutations. Genetic redundancy could also limit gene identification. If defense activation pathways are very short, few additional loci may exist to be identified. Genes that function after a branch point in defense induction and control a subset of downstream defense responses could easily escape detection if their contribution to resistance is only quantitative or partial. Lastly, recovery of a relevant class of mutants might be precluded by the particular screening method used. Screening of larger populations might be all that is required to uncover mutations in other loci of the *avrRpt2-RPS2* pathway. As an alternative, the present mutational study addressed these issues through a modified screening procedure (Yu et al. 1998) that was sensitive to partial-loss-of-function mutations. This screen monitored the presence and timing of the hypersensitive response (HR).

The HR is a programmed cell death process intimately associated with the defense response mediated by plant resistance genes and pathogen avirulence genes (Bent 1996; Dangl et al. 1996; Goodman and Novacky 1994; Hammond-Kosack and Jones 1996; Richberg et al. 1998). Beneficial roles of the HR include the elimination of host cells that support pathogen growth, the release of antimicrobial compounds, and the release of signals that activate defense responses in neighboring and distant host cells. The HR is not always required for effective gene-for-gene resistance (Bendahmane et al. 1999; Goulden and Baulcombe 1993; Lehnackers and Knogge 1990; Ori et al. 1997; Schiffer et al. 1997; Yu et al. 1998). However, the above-noted benefits of HR cell death are apparently important in many gene-for-gene interactions, and useful information is expected from further study of the relationship between HR cell death and activation of host defenses.

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We recently reported the identification of *Arabidopsis* *DND1* (Yu et al. 1998). *Arabidopsis* plants homozygous for the recessive *dnd1-1* mutation exhibit a “defense, no death” phenotype, in which plants exhibit strong gene-for-gene resistance to avirulent *P. syringae* despite the virtual absence of HR cell death. Mutant *dnd1* plants also exhibit a degree of constitutive resistance to virulent *P. syringae*, *Peronospora parasitica*, and *Tobacco ringspot virus*, similar to the defense observed in plants induced for systemic acquired resistance. This constitutive resistance is quantitatively less effective than the inducible gene-for-gene resistance. The *dnd1* mutant, along with some *Arabidopsis* *lsd*, *acd*, *cpr*, and *cim* mutants, fits into a larger class of mutated lines that exhibit a dwarfed rosette, elevated levels of salicylic acid (SA), and constitutively heightened resistance to virulent pathogens. These mutants differ from each other by the presence or absence of lesion mimicry, by their HR phenotypes, by leaf shape, glabrousness and other morphological phenotypes, and by the different loci that are responsible for the mutant traits.

The present report describes additional *Arabidopsis* mutants isolated based on their altered HR in response to *P. syringae* that express *avrRpt2*. Like *dnd1*, many of these mutants exhibit elevated resistance to virulent pathogens as well as a reduced HR in response to avirulent *P. syringae*.

RESULTS

Mutant screen.

A mutational approach was pursued to identify components of the *avrRpt2-RPS2* disease resistance pathway in *Arabidopsis*. Plants were inoculated with *P. syringae* that express *avrRpt2* (Dong et al. 1991; Whalen et al. 1991). Artificially high titers of pathogen were used to induce confluent, macroscopically observable HR cell collapse (Klement et al. 1964). Screens for loss of HR had previously been used to identify mutant alleles of *RPS2* (Yu et al. 1993), but for the present work we designed a new method that allowed high throughput screening with detection of intermediate or delayed-HR phenotypes (see also Kunkel et al. 1993; Yu et al. 1998). A delayed HR can be difficult to detect if plants are inoculated with virulent *P. syringae* that express an *avr* gene because at high inoculum titers the virulence of these strains leads to

killing and necrosis of susceptible plant cells roughly 36 to 48 h after inoculation. Accordingly, a *P. syringae* pv. *glycinea* strain (Psg R4) that does not cause disease on *Arabidopsis* was used as a vehicle for delivery of the HR-inducing signal controlled by *avrRpt2* (Yu et al. 1993, 1998). A bulk inoculation method was designed (see Materials and Methods) that treated some but not all leaves of multiple plants. The goal was to avoid induction of a systemic HR that could eventually kill plants that exhibit an intermediate or delayed HR. Ethylmethane sulfonate (EMS) was chosen as the mutagen to enhance the likelihood of generating partial-loss-of-function alleles.

Out of approximately 11,800 M_2 plants screened, 209 candidate lines initially displayed a delayed or no HR. Delayed HR was defined as failure to develop the HR within 24 h but presence of the HR within 40 h. Candidate plants were transplanted to larger pots, and re-inoculated with Psg R4 *avrRpt2*⁺ 3 weeks after transplantation. Forty-two lines were then saved after giving a delayed HR or no response to Psg R4 *avrRpt2*⁺ in the re-test. When self-progeny of these were planted for further study, 23 lines displayed a typical HR 24 h after inoculation and were not studied further. The M_3 progeny from 12 other mutants exhibited little or no HR 40 h after inoculation. Seed was saved from individual HR[−] M_3 plants from these lines. In order to eliminate the possibility of contamination by the mutant *rps2-201* (which was planted in the original screen as a control), a pair of *rps2-201*-specific polymerase chain reaction (PCR) primers was used to detect the *rps2-201* allele in genomic DNA of the HR[−] mutants. Six lines contained an *rps2-201* allele and were excluded from subsequent work. The other six HR[−] mutants, Y3, Y5, Y15, Y16, Y23, and D25, did not contain the *rps2-201* allele and were saved for further analysis (Table 1).

A weak or intermediate HR, or a mix of HR and no HR, was observed in the progeny of the final seven mutants, N8, N12, N13, N16, D8, D15, and D24 (Table 1). These lines also did not contain the *rps2-201* allele. Whenever the HR[−] phenotype was observed in some but not all progeny derived from self-fertilization of these mutants, seeds from plants that had the clearest HR[−] phenotype were saved and re-tested in the next generation. All of these “intermediate-HR” mutant lines retained an intermediate HR phenotype in the M_4 generation except N8, which was apparently isolated as an *RPS2/rps2*

Table 1. HR (hypersensitive response), complementation, and growth morphology phenotypes of altered-HR mutants

Mutant group	Name	<i>avrRpt2</i>		Complementation of <i>rps2-201C</i>	Other phenotypes
		Phenotype	HR data ^a		
Controls	Col-0	HR ⁺	4.7 ± 0.1	Yes	Wild-type parent
	Col-0 <i>rps2-201C</i>	No HR	0.1 ± 0.0	No	
I	N8	No HR	0.0 ± 0.0	No	
	D25	No HR	0.0 ± 0.0	No	
II	N12	Mixed HR	3.0 ± 0.5	Yes	
	N13 (<i>ihr1</i>)	Mixed HR	3.2 ± 0.2	Yes	
	N16	Mixed HR	1.5 ± 0.3	Yes	
	D8	Mixed HR	3.9 ± 0.2	Yes	Slight leaf narrowing
	D24	Mixed HR	4.2 ± 0.2	Yes	
III	Y16 (<i>dnd1</i>)	No HR	0.2 ± 0.2	Yes	Dwarf
	Y3 (<i>dnd2</i>)	No HR	0.6 ± 0.2	Yes	Dwarf
	Y15	Weak HR	2.2 ± 0.3	Yes	Semi-dwarf
IV	Y23	No HR	0.5 ± 0.3	Yes	Spontaneous lesions, dwarf
V	Y5	No HR	1.7 ± 0.7	Yes	Dwarf, low fertility
	D15	No HR	2.2 ± 0.3	Yes	Delayed development, low fertility

^a Values are mean ± SE of HR severity (0 = no HR, 5 = extensive HR). All values are significantly different from HR data for Col-0 (*t* test, *P* < 0.05).

heterozygote (see below). In summary, 13 mutant lines were saved for further study (Table 1). Our isolation and characterization of Y16, a line mutated in the *DND1* gene, was previously described (Yu et al. 1998).

RPS2 complementation tests.

Crosses were made between many of the mutants and an *rps2* mutant (Col-0 *rps2-201/rps2-201*). The Col-0 *rps2-201* line was used as a pollen donor and true crosses were verified by PCR to test genomic DNA from F₁ leaf samples for presence of the *rps2-201* allele. The F₁ individuals from each cross were challenged with Psg R4 *avrRpt2*⁺, and an HR was observed in all F₁ except those from crosses with mutants N8 and D25. The N8 and D25 lines were considered probable *rps2* mutants and were placed in "Group I" (Table 1). However, the possibility remained that N8 and/or D25 carry a dominant suppressor of the HR. Therefore, F₂ plants from crosses between Col-0 *rps2-201* and N8 or D25 were examined for the HR in response to Psg R4 *avrRpt2*⁺. No HR was observed in any individuals from these F₂ populations. This was not consistent with the 3:13 ratio of HR⁺:HR⁻ plants that might have been expected if N8 or D25 carried a dominant suppressor. Mutants N8 and D25 failed to complement the *rps2-201* mutant; therefore, we concluded that mutants N8 and D25 carry *rps2* mutations.

Group II: Intermediate-HR mutants.

We identified five mutants that displayed an intermediate-HR phenotype (Table 1, Group II). As an example of this phenotype, inoculation of three leaves on a given plant often produced one or two leaves showing extensive HR tissue collapse while the other one or two leaves failed to show any HR in response to the avirulent pathogen. HRs of intermediate severity were also present. This type of leaf-to-leaf variation was also observed when vacuum infiltration was used to inoculate plants. In contrast, over 95% of all leaves in control compatible and incompatible interactions gave no HR or full HR, respectively. Significantly, the mutants in Group II did not exhibit any obvious dwarfing or lesion-mimic phenotypes. Mutants N13 and D8 received the greatest attention in subsequent studies.

Specificity for different avirulence genes.

To address whether the Group II mutants N13 and D8 were altered only in the *avrRpt2-RPS2* pathway, we inoculated plants with Psg R4 strains carrying other *avr* genes. The mutants in this study were derived from Col-0 plants that carry a functional *RPM1* gene, and the *avrRpm1* and *avrB* genes elicit a strong defense response in plants expressing *RPM1*. When mutant N13 was tested, an intermediate-HR was observed in response to both Psg R4 *avrRpm1*⁺ and Psg R4 *avrB*⁺ (Table 2). Similar results were obtained with another *P. syringae* strain, Psg R4 RUU1, and either *avrRpt2*, *avrRpm1*, or *avrB* (data not shown). These data suggest that the mutation in the N13 line affects a shared component of different *avr-R* gene pathways.

Mutant D8 also was tested with *P. syringae* carrying different avirulence genes. The HR scores observed in D8 in response to Psg R4 that express either *avrRpm1* or *avrB* were not significantly different from the HR of Col-0 in response to these pathogen strains (Table 2).

Growth of *P. syringae* in mutants N13 and D8.

The HR is closely associated with gene-for-gene resistance, and loss of HR generally indicates loss of resistance to pathogens that express the *avr* gene. To test whether the partially compromised HR in mutants N13 and D8 was associated with defective limitation of bacterial growth, the growth of *P. syringae* pv. *tomato* (Pst) was monitored in both mutants. Plants were inoculated with virulent Pst DC3000 or with Pst DC3000 that express the cloned avirulence genes *avrRpt2*⁺, *avrB*⁺, or *avrRps4*⁺. Low initial doses of bacteria (5×10^4 CFU per ml) were applied. Mutant N13 and wild-type Col-0 displayed a similar degree of resistance to *P. syringae* that express either *avrRpt2* or *avrB* (Fig. 1). For avirulence gene *avrRps4* and resistance gene *RPS4*, the resistance response of ecotype Col-0 is quantitatively less effective than the response of other ecotypes such as Po-1, and is less strong than the response of Col-0 to *P. syringae* that express avirulence genes such as *avrRpt2* or *avrB*. (Hinsch and Staskawicz 1996; Fig. 1). When N13 plants were inoculated with Pst DC3000 that express *avrRps4*, we discovered an increase rather than decrease in resistance. A 10-fold reduction in Pst DC3000 *avrRps4*⁺ population size was observed in N13 relative to Col-0 (Fig. 1).

The above result with *avrRps4* may be related to the separate finding that non-race-specific resistance to virulent *P. syringae* was also enhanced in the N13 line. Four days after inoculation, populations of virulent Pst DC3000 (no cloned *avr* gene) were roughly 10-fold lower in N13 relative to wild-type Col-0 (e.g., Fig. 1; reductions were twofold to 40-fold in three independent experiments). In contrast to many previously described Arabidopsis *cpr*, *cim*, *lsd*, *acd*, and *dnd* mutants (Bowling et al. 1994; Dietrich et al. 1994; Dong 1998; Greenberg et al. 1994; Richberg et al. 1998; Yu et al. 1998), this elevated resistance phenotype was present in N13 despite the absence of any discernible reduction in plant size, fecundity, or leaf health.

Mutant D8 also exhibited strong resistance to Pst DC3000 that express *avrRpt2*, *avrB*, or *avrRps4* (Fig. 1). There was no significant difference between mutant D8 and Col-0 in the degree of resistance to Pst DC3000 *avrRpt2*⁺ or *avrB*⁺ (Fig. 1). As for N13, elevated resistance to Pst DC3000 *avrRps4*⁺ was observed in mutant D8 (Fig. 1). Slightly less resistance to virulent Pst DC3000 was detected in mutant D8 than in mutant N13, but resistance to Pst DC3000 was still stronger than the wild-type Col-0 (e.g., Fig. 1; ninefold, twofold, and ninefold differences between D8 and Col-0 were observed in three independent experiments). Therefore, the weaker resistance in D8 relative to

Table 2. Hypersensitive response (HR) of selected mutants to *Pseudomonas syringae* expressing different avirulence genes^a

Mutant	Avirulence gene		
	<i>avrRpt2</i>	<i>avrRpm1</i>	<i>avrB</i>
Col-0	4.7 ± 0.1	4.0 ± 0.5	4.5 ± 0.3
N13 (<i>ihrl</i>)	3.2 ± 0.2 ^b	2.4 ± 0.5 ^c	2.6 ± 0.6 ^b
D8	3.9 ± 0.2 ^b	3.6 ± 0.3	3.4 ± 0.1
Y15	2.2 ± 0.3 ^b	3.3 ± 0.3	Not tested
Y3 (<i>dnd2</i>)	0.6 ± 0.2 ^b	0.0 ± 0.0 ^b	0.4 ± 0.2 ^b
Y23	0.5 ± 0.3 ^b	1.4 ± 0.9 ^b	2.6 ± 1.1 ^b

^a Values are mean ± SE of HR severity (0 = no HR, 5 = extensive HR). Data for *avrRpt2* are the same as those from Table 1.

^b Significantly different from Col-0 inoculated with same pathogen strain (*t* test, *P* = 0.05).

^c For *t* test comparison to Col-0, *P* = 0.088.

N13 correlated with the weaker loss of HR in D8 relative to N13. As for N13, D8 exhibited this enhanced resistance with no discernible defects in plant size, fecundity, or leaf health.

Disease assay.

To further characterize the resistance response in N13 and D8, we inoculated these lines with either Pst DC3000 *avrRpt2*⁺ or Pst DC3000 and monitored macroscopic disease symptom development. Col-0, N13, and D8 were immersed into 0.01% Silwet L-77 surfactant carrying Pst DC3000 *avrRpt2*⁺ or Pst DC3000, and disease symptoms were scored 4 days after inoculation. The degree of disease was rated on a 0 to 5 scale (0 = no lesions, 5 = confluent necrosis and chlorosis). Disease assay experiments were repeated on three separate dates with consistent results. Col-0, N13, and D8 all showed resistance to Pst DC3000 *avrRpt2*⁺ (average score < 0.5). Severe disease lesions were observed in Col-0 inoculated with Pst DC3000 (average score = 5). On the other hand, N13 and D8 exhibited some resistance to Pst DC3000 (average score ≈ 2). This result was consistent with the elevated resistance to Pst DC3000 observed in the bacterial growth experiments (Fig. 1).

Genetic analysis of N13 defines the *IHR1* locus.

Mutant N13 displayed an intermediate HR, or occasionally no HR, in response to Psg R4 *avrRpt2*⁺. To assess dominance and to remove other unrelated mutations, N13 was backcrossed to wild-type Col-0. F₁ plants from N13 × Col-0

crosses exhibited a consistent HR within 24 h in response to Psg R4 *avrRpt2*⁺, suggesting that N13 carries a recessive mutation. In an F₂ population of N13 × Col-0, the segregation ratio for HR⁺: intermediate-HR: HR⁻ was 22:10:9. However, F₂ scoring was likely to be less reliable given the variable nature of the intermediate-HR phenotype and the limited number of leaves that could be assayed on each single plant. This suspicion was confirmed in progeny testing of a limited number of F₃ families. For example, the F₃ progeny from two independent F₂ plants that had been scored as HR⁻ showed 19:25:3 and 18:25:6 ratios for HR⁺: intermediate-HR: HR⁻. Therefore, all subsequent conclusions regarding HR segregation data were based on F₃ progeny test data.

For further genetic analysis of the N13 mutant, crosses were made between N13 and wild-type plants of the No-0 ecotype. A strong HR was observed in the F₁ plants. In the F₂, segregation of the HR phenotype closely resembled a 3:1 ratio (HR⁺: intermediate-HR or HR⁻ = 126:48; $\chi^2 = 0.62$, $P = 0.43$). F₃ segregation patterns were also consistent with a single-gene model of inheritance. In F₃ progeny tests, vacuum infiltration was used to apply Psg R4 *avrRpt2*⁺ to 12 to 18 plants per F₃ family. These F₃ families were subsequently scored on a 0 to 3 scale, with 0 = No HR (similar to Col-0 *rps2-201C* controls), 1 = infrequent HR (similar to N13 controls), 2 = majority of plants with HR on most leaves, and 3 = HR⁺ leaves on all plants (similar to wild-type Col-0 controls). Of 100 F₃ families that were tested once, 70 families had a score of 2 or 3 and 30 families had a score of 1. This 70:30 ratio was consistent with a 3:1 ratio ($\chi^2 = 1.33$, $P = 0.25$). An additional 57 F₃ families were tested twice. Of those families, 46 exhibited a prevalent HR in at least one test while the other 11 gave infrequent HR in both tests (scores of 1 and ≤1). This 46:11 ratio was consistent with 3:1 segregation ($\chi^2 = 0.98$, $P = 0.32$). The data were still consistent with a 3:1 ratio even if families that were scored once as a “1” and once as a “2” in the two tests were shifted into the reduced-HR category ($\chi^2 = 3.09$, $P = 0.08$). The above data therefore indicate that the N13 line carries a recessive (and incompletely penetrant) mutation at a single Mendelian locus that causes a partial or intermediate loss of HR in response to *P. syringae* that express *avrRpt2*. We chose the gene symbol *ihrl* for the mutant locus in N13, indicating the phenotype of “intermediate hypersensitive response.”

Genetic analysis of additional Group II mutants.

Although our original screen succeeded in identifying lines with a reproducible, reduced-HR phenotype, the intermediate or intermittent nature of the HR defect in the other Group II mutants presented a significant challenge for genetic analysis. Mutants D8, N12, and N16 were crossed to wild-type Col-0 and/or to ecotype No-0. Studies with the resulting F₁, F₂, and F₃ progeny gave results that were not inconsistent with the segregation of single recessive loci controlling the reduced-HR phenotype (data not shown). However, the level of uncertainty in the scoring of individual F₂ or F₃ lines as HR⁺ or reduced-HR indicated that genetic mapping would be unreliable without extensive replications, and no further genetic analysis of these lines was attempted.

Group III: HR-/dwarf mutants.

The Group III mutants Y3 and Y16 shared many phenotypes, including a dwarfed rosette morphology and near-

Fig. 1. Growth, in *Arabidopsis* Col-0 and mutants N13 (*ihrl*) or D8 plants, of *Pseudomonas syringae* pv. *tomato* DC3000 that express different *avr* genes. Six-week-old plants were inoculated with Pst DC3000 carrying pVSP61 (empty plasmid vector, “vir”) or pVSP61 carrying the designated avirulence gene. Filled symbols: N13 (left panels) or D8 (right panels); open symbols: Col-0. Col-0 data from left panels are repeated in right panels to facilitate comparison.

complete loss of the HR in response to *P. syringae* expressing *avrRpt2*, *avrRpm1*, or *avrB*. (Tables 1 and 2, Fig. 2; see also Yu et al. 1998). Despite the HR⁻ phenotype of Y3 and Y16, they continued to exhibit gene-for-gene resistance (Yu et al. 1998; I.-c. Yu, R. K. Smith, K. A. Fengler, and A. F. Bent, *manuscript in preparation*). Accordingly, these lines were termed “defense, no death” (*dnd*) mutants and lines Y16 and Y3 were re-named *dnd1* and *dnd2*, respectively. The *dnd1* mutant received the bulk of our earlier attention, and was previously described (Yu et al. 1998). Additional phenotypic and genetic analyses of the *dnd1* and *dnd2* lines will be reported separately (I.-c. Yu, R. K. Smith, K. A. Fengler, and A. F. Bent, *manuscript in preparation*).

Y15 exhibited a less severe phenotype, with a semi-dwarfed rosette that was larger than that of Y16 (*dnd1*) or Y3 (*dnd2*), and with an HR⁻ phenotype that was more consistent than most of the Group II intermediate-HR mutants but less consistent than in *dnd1* or *dnd2*. Y15 could have been placed in Group II but was placed in Group III because of the mild dwarfing of leaves/rosettes. No lesion mimic phenotype was observed in noninoculated Y15 leaves following gross inspection or microscopy of trypan blue-stained samples (data not shown).

Differences in the leaf morphology of Group III mutants extended beyond the dwarf size. Leaves of *dnd1*, *dnd2*, and Y15 often became slightly rolled or curled with age, and *dnd2* in particular often developed chlorosis at the ends (tips) of leaves during later stages of growth. Plants were otherwise quite healthy in appearance and did not exhibit other obvious developmental defects.

Characterization of Y15.

When the growth of *P. syringae* pv. *tomato* was monitored in Y15, these plants resembled other Group II and Group III mutants in two respects. First, despite the reduced HR, the Y15 plants continued to exhibit strong restriction of the growth of avirulent pathogens expressing *avrRpt2* or *avrRpm1* (Fig. 3). Second, Y15 plants exhibited enhanced resistance to virulent Pst DC3000 (Fig. 3; note that in a repeat of the experiment of Figure 3, the population size of virulent DC3000 4 days after inoculation was only 40-fold lower in Y15 relative to Col-0). Elevated resistance to virulent Pst DC3000 was also observed in surfactant-dip disease assays. In contrast to

the *ihrl* and D8 lines, for which mild disease symptoms were observed (presented above), virtually no disease symptoms were observed on Y15 plants in two separate surfactant dip experiments with virulent Pst DC3000. As might be expected, Y15 plants were highly resistant to Pst DC3000 *avrRpt2*⁺ in the surfactant dip assay. When the HR to *P. syringae* expressing different *avr* genes was monitored by the syringe inoculation procedure, Y15 resembled the intermediate-HR mutants rather than the *dnd* mutants. Significant reduction of HR severity was observed in response to *P. syringae* *avrRpt2*⁺, but the reduction in HR score in response to *P. syringae* that express *avrRpm1* was not statistically significant (Table 2).

Northern (RNA) blot transcript analysis was performed with mutant Y15 to relate the intermediate-HR and elevated-resistance phenotypes to alterations in defense-related gene expression. We found that Y15 leaves exhibited elevated PR-1 transcript levels, relative to wild-type Col-0, in the absence of any inoculation with pathogen (Fig. 4; similar data obtained in repeat experiments). We also observed that pathogen-inducible PR-1 gene expression was potentiated in Y15. Inoculation with virulent or avirulent *P. syringae* caused an increase in PR-1 gene expression to levels that were significantly above those observed in Col-0 (Fig. 4). In contrast, β -glucanase expression was largely unaffected in Y15; RNA levels were within twofold of wild type in all treatment conditions in multiple experiments (data not shown).

In light of the elevated disease resistance and PR-1 gene expression in Y15, we investigated the level of SA in Y15 plants. There was a clear increase in SA relative to wild-type Col-0 (Fig. 5A). Interestingly, the concentrations of SA and SA-glucoside in Y15 were roughly one-half the levels observed in *dnd1* and *dnd2* plants from the same experiments (data not shown), which correlates with the less penetrant HR⁻ and disease resistance phenotypes of Y15 relative to *dnd1* and *dnd2*. When a 35S-*nahG* (salicylate hydroxylase) construct was introduced into Y15 to remove SA, the elevated disease resistance of Y15 plants was eliminated and plants were instead more susceptible than wild type (Fig. 5B).

In genetic analysis of Y15, F₁ plants of Col-0 $\times$ Y15 gave a clear HR⁺ phenotype, suggesting recessive behavior of the mutation in Y15. As with the other intermediate-HR mutants, analysis of Col $\times$ Y15, No-0 $\times$ Y15, and other F₂ populations and F₃ progeny families revealed segregation of apparent true-

Fig. 2. Morphology of dwarfed mutant lines. Typical 5-week-old plants are shown to indicate relative sizes and appearance.

Fig. 3. Growth in Arabidopsis Col-0 and mutant Y15 plants of *Pseudomonas syringae* pv. *tomato* DC3000 carrying different *avr* genes. Six-week old plants were inoculated with Pst DC3000 carrying pVSP61 (plasmid vector only) or pVSP61 carrying the designated avirulence gene. Filled symbols: Y15; open symbols: Col-0.

breeding HR⁺ and true-breeding reduced-HR lines. However, the reliability of the HR scoring inhibited genetic analysis at the level of precision required for genetic mapping of the mutant locus. For No-0 × Y15 F₃ families we also attempted to score segregation based on the partial-dwarf morphology, and based on the enhanced resistance to virulent Pst DC3000 or *P. syringae* pv. *maculicola* 4326. However, these phenotypes were also sufficiently intermediate or quantitative to inhibit straightforward scoring, and we concluded that extensively replicated testing would be required for reliable assignment of phenotypes to most F₃ families.

Resistance to virulent *Peronospora parasitica*.

To examine the possibility that the intermediate-HR mutants may have an altered response to virulent pathogens other than *P. syringae*, the oomycete pathogen *Peronospora parasitica* was used to test the resistance of the *ihr1* and Y15 mutant lines. These mutants, as well as Col-0 and Ler-0 controls, were inoculated with *P. parasitica* Noco2 by spraying of 9-day-old seedlings. As expected (Aarts et al. 1998), Ler-0 exhibited resistance and Col-0 exhibited susceptibility to this *P. parasitica* strain. The *ihr1* plants resembled Col-0 in being susceptible to *P. parasitica*, with only slightly lower fungal proliferation observed. In contrast, Y15 plants exhibited partial but clearly discernible enhanced resistance in three independent experiments. The Noco2 strain did proliferate and form haustoria on multiple cells in Y15 leaves, but this occurred at a reduced rate relative to Col-0 controls. Sporulation levels on Y15 were 41 and 66% of wild-type Col-0 controls in the two experiments in which spore counts were taken.

Group IV: HR⁻/dwarf/lesion-mimic mutant.

Mutant line Y23 resembled the *dnd* mutants in exhibiting a dwarfed rosette morphology and a failure to develop extensive hypersensitive cell death in response to *P. syringae* that express *avrRpt2* (Table 1, Fig. 2). However, mutant Y23 and its progeny developed spontaneous disease lesion-like necrotic

Fig. 4. PR-1 RNA levels in wild-type Col-0 and Col-0 mutant Y15 leaf tissue. Plants were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 carrying pVSP61 (plasmid vector only) or pV288 (*avrRpt2*⁺). Hybridization of PR-1 probe was quantified with a phosphorimaging system; all data points were normalized to the level of constitutive β-ATPase transcript in the same sample, and then further normalized such that expression levels for noninoculated Col-0 = 1.

leaf flecks in the absence of inoculation with pathogen. This is similar to some *lsd* ("lesions simulating disease") and *acd* ("accelerated cell death") *Arabidopsis* lines previously described (Dietrich et al. 1994; Greenberg et al. 1994; Richberg et al. 1998). Randomly located, isolated lesions had typically formed on lower leaves of Y23 plants by the time these plants were 4 weeks old. In contrast to the "propagation class" lesion-mimic mutants that lack the ability to stop lesion spread and ultimately display death of entire leaves and plants (Dangl et al. 1996), Y23 instead resembled the "initiation class" mutants and formed lesions of a limited size that did not engulf entire leaves. Moreover, the lesions were not enhanced by intensive light or by inoculation with avirulent *P. syringae*.

When bacterial growth was monitored within Y23 leaves, the plants exhibited enhanced resistance to both virulent and avirulent *P. syringae* (Fig. 6 and data not shown). PR-1 and β-glucanase transcript levels were constitutively elevated in Y23 (data not shown). This is similar to the constitutive resistance phenotype reported not only for other *Arabidopsis* lesion-mimic mutants, but also for *Arabidopsis cpr*, *cim*, *dnd*, and *ihr* mutants (Bowling et al. 1994; Dietrich et al. 1994; Dong 1998; Greenberg et al. 1994; Richberg et al. 1998; Yu et al. 1998; the present work). However, of the published lesion-mimic mutants, only Y23 and *acd6* have been reported to exhibit an HR⁻ phenotype (Rate et al. 1999).

Fig. 5. A, Elevated salicylic acid (SA) levels in mutant Y15 as opposed to wild-type Col-0. B, Elimination of enhanced resistance to growth of *Pseudomonas syringae* upon expression of salicylate hydroxylase. Free SA levels were determined with noninoculated leaf tissues. Population densities of *P. syringae* pv. *tomato* DC3000 in leaves were determined 3 days after inoculation. Samples a, b, c, and d are four independently derived Y15 transgenic lines expressing a 35S-*nahG* construct.

Fig. 6. Growth in *Arabidopsis* Col-0 and mutant Y23 plants of *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 or DC3000 expressing *avrRpm1*. Six-week-old plants were inoculated with Pst DC3000 carrying pVSP61 (plasmid vector only) or pavrRpm1 (*avrRpm1*⁺). Filled symbols: Y23; open symbols: Col-0.

F₁ plants from a Y23 × Col-0 cross were normal in appearance and were HR⁺ in response to *P. syringae* expressing *avrRpt2*. In a Y23 × Col-0 F₂ population, the spontaneous lesion phenotype segregated in a 3:1 ratio (no lesion: lesion = 79:23; $\chi^2 = 0.33$; $P = 0.57$). These data indicate that the lesion-mimic trait of Y23 is controlled by a single recessive gene. However, the segregation ratio for plant size in the above Y23 × Col-0 F₂ population was not 3:1 (wild-type: dwarf morphology = 93:9; χ^2 for 3:1 = 14.2; $P < 0.001$). The presence of spontaneous lesions on F₂ plants of wild-type size suggests that there is incomplete penetrance of the dwarfing phenotype in lines homozygous for the *lsd/acd*-type mutation from line Y23.

The HR-suppression and elevated-resistance phenotypes of Y23 were temporally correlated with the onset of lesion formation. Unlike the 6- to 8-week-old Y23 plants discussed above and presented in Tables 1 and 2, the HR was readily detectable on younger Y23 plants (less than 4 weeks old) that were inoculated with avirulent *P. syringae* before spontaneous lesions had developed. When young Y23 plants with few or no spontaneous lesions were inoculated with the virulent *P. parasitica* strain Noco2, the plants resembled Col-0 in being highly susceptible to infection. This is in contrast to the *dnd* mutants, which exhibited resistance to virulent *P. parasitica* Noco2 and loss of the HR induced by avirulent *P. syringae* even in 3- to 4-week-old plants (Yu et al. 1998; I.-c. Yu, R. K. Smith, K. A. Fengler, and A. F. Bent, *manuscript in preparation*). Even in 6- to 8-week-old Y23 plants (after lesion formation), the reduction of HR in response to *P. syringae* expressing *avrRpm1* or *avrB* was more quantitative or partial than for the *dnd* mutants (Table 2).

Group V: Low fertility HR⁻ mutants.

The Group V mutants, Y5 and D15, exhibited a reasonably clear HR⁻ phenotype in progeny tests (Table 1). Genetic studies with these lines were initiated by backcrossing to Col-0 and outcrossing to No-0. However, the Y5 and D15 lines exhibited severely compromised fertility and received little further study.

DISCUSSION

To identify unknown components of the *avrRpt2*-*RPS2* disease resistance pathway of Arabidopsis, we initiated a screen for mutants exhibiting a partial or complete loss of HR in response to *P. syringae* that express the *avrRpt2* gene. The screen was designed to overcome the inherent limitations of many mutational studies by allowing identification of genes that make quantitative contributions to resistance and/or genes that are lethal when fully inactive. Mutant screens often identify unexpected variants of the desired phenotype. The present screen not only produced *rps2* mutants defective in resistance to *P. syringae* that express *avrRpt2*, but also detected mutants that are defective in the HR but competent or even enhanced for disease resistance.

Thirteen mutant lines were isolated and then divided into five groups. The first group contained two new *rps2* mutants. Group II plants, including N13 (*ihr1*), displayed an intermediate-HR phenotype in which HR⁺, intermediate HR, and HR⁻ reactions could be observed on different leaves of the same plant. Group III contained defense-no-death (*dnd*) mutants

that exhibited dwarfed growth and a more complete loss of HR, yet carried out successful gene-for-gene disease resistance. The sole mutant in Group IV was a dwarf, HR⁻ mutant that developed spontaneous lesions prior to inoculation with pathogen. Interestingly, plants from Groups II, III, and IV also exhibited partial elevation of resistance to virulent *P. syringae*. Group V was composed of HR⁻ plants that received little study because they produced very few seeds. The low fertility of the Group V mutants may be caused by irrelevant secondary mutations. Alternatively, these lines may demonstrate an important mutant class: highly relevant defense-related genes that are difficult to identify and study by mutation due to associated deleterious phenotypes.

The “partial function” Y15, *ihr1*, and other Group II mutants that we isolated exhibited HR⁺, intermediate-HR, and HR⁻ phenotypes on different leaves of the same plant. The mixed or intermittent HR behavior of these lines suggests that the relevant mutations have shifted pathogen response pathways closer to some threshold for HR cell death activation. All inoculated leaves on a given plant received a similar dose of pathogen, but the mutations apparently compromised HR pathways in a way that caused subtle differences between leaves to result in very dramatic differences in HR phenotypes. Gene-for-gene disease resistance remained effective despite this altered HR phenotype (Figs. 1 and 3). We have observed a similar intermediate- or mixed-HR phenotype in transgenic Arabidopsis lines carrying *RPS2* alleles with conservative amino acid substitutions in the nucleotide binding site but, unlike the present mutants, those *rps2* mutant lines exhibit less effective restriction of pathogen growth (E. Baima, D. Banerjee, and A. Bent, *unpublished data*). Arabidopsis *phx/lsd5* double mutants that suppress cell death have been characterized by Morel and Dangl (1999) but those lines also exhibit compromised restriction of avirulent *P. syringae*.

It is worth noting that the partial-loss-of-function phenotypes of some of the mutants reported in this paper may be due to subtle or severe mutations. For example, the *ihr1* mutant may carry a total-loss-of-function allele that exerts a subtle effect on resistance, or *IHR1* may play a central role in transducing resistance signals from different *avr/R* gene pathways and the *ihr1* mutant may carry a subtle mutation in this gene.

Because *ihr1* exhibits similar alteration in HR and similar retention of resistance in response to different avirulent pathogens, it is likely that the *ihr1* mutation exerts an effect on signal transduction processes downstream of the events mediated by specific *avr* and *R* (resistance) gene products. In contrast, mutants D8 and Y15 displayed an altered *avrRpt2*-*RPS2* HR, but the intermediate HR response was statistically indistinguishable from wild-type for the *avrRpm1*-*RPM1* pathway. However, this difference may or may not indicate that the mutations in D8 and Y15 alter a gene with functional specificity for the *avrRpt2*-*RPS2* pathway. *RPM1* mediates an earlier/stronger HR than *RPS2* in wild-type plants (Ritter and Dangl 1996; I.-c. Yu and A. F. Bent, *unpublished data*). It may be the case that loss or inhibition of the HR in the D8 and Y15 mutants is too weak to alter the *RPM1*-mediated response.

Significantly, the *ihr1*, D8, and Y15 mutants exhibited a modest but highly reproducible increase in resistance to virulent *P. syringae* (Figs. 1 and 3; and data not shown). While *ihr1*, D8, and Y15 still developed some disease symptoms

(necrotic lesions and chlorosis), the extent of disease was substantially lower than in Col-0. Y15 also exhibited elevated resistance to a second pathogen, the virulent *P. parasitica* strain Noco2. The partial elevation of resistance in Y15 correlated with elevated PR-1 expression and elevated levels of SA. The dependence of resistance on SA was shown in Y15 *nahG*⁺ lines.

In addition to the enhanced resistance to virulent *P. syringae*, elevated resistance in the *avrRps4-RPS4* pathway was observed. Wild-type Col-0 plants only exhibit weak restriction of the growth of *P. syringae* that express *avrRps4* and only a weak HR in response to these strains, providing a particularly sensitive system with which to detect altered defense responses (Hinsch and Staskawicz 1996; and this work). The enhanced resistance in the *ihr1* and D8 mutant lines both to virulent *P. syringae* and to *P. syringae* that express *avrRps4* suggests a relationship between enhanced resistance to virulent pathogens and potentiation of gene-for-gene signaling. These data provide further evidence of the overlap that has been observed between gene-for-gene and more general defense responses (Delaney et al. 1994; Richberg et al. 1998; Shirasu et al. 1997). The HR apparently reinforces or enhances defense activation in plant tissues (Alvarez et al. 1998; Dangl et al. 1996), but in intermediate-HR mutants the elevated constitutive defenses may substitute for the HR and foster an analogous enhancement of gene-for-gene defenses.

Unlike previously reported Arabidopsis lines with elevated resistance such as many of the *cpr*, *cim*, *lsd*, and *acd* mutants and our *dnd* mutants (Dong 1998; Richberg et al. 1998; Yu et al. 1998), the elevated resistance in *ihr1* and D8 occurred without dwarfing of the plants. This may be due to a significant difference in the mechanism of defense activation in *ihr1* and D8 as opposed to the previous mutants. Alternatively, the absence of dwarfing may be a quantitative phenomenon that correlates with the weaker elevation of nonspecific resistance in *ihr1* and D8 as opposed to the dwarfed mutants, and that correlates with the less complete loss of HR in the *ihr1* and D8 mutants. Consistent with this correlation, Y15 plants did exhibit partial dwarfing, and also exhibited heightened resistance to *P. parasitica* that was not observed in the *ihr1* line. Despite the intentional design of our mutant screen to detect intermediate phenotype mutants, difficulties with reliable phenotypic scoring of segregating progeny made precise genetic analysis of these partial-function mutants a challenging task. However, strong agricultural biotechnology opportunities could be revealed through identification and study of genes that elevate resistance without associated penalties in plant growth and productivity.

In contrast to *ihr1*, D8, and the other Group II mutants, the *dnd* (Y3 and Y16) and *lsd/acd* (Y23) mutants that we isolated exhibited a more complete loss of HR, an obvious dwarfed phenotype, and a more strongly elevated resistance against virulent *P. syringae*. These similarities between our *dnd* and *lsd/acd* mutants are interesting in light of their obviously different lesion-mimic phenotypes. It is also interesting that many previously isolated dwarf/elevated resistance mutants (including many *cpr*, *cim*, *lsd*, and *acd* mutants) still generate HR cell death in response to avirulent *P. syringae*, while the HR is largely absent in the *dnd* and *lsd/acd* mutants that we isolated. These differences imply that the metabolic alterations caused by these various mutations, while partially overlapping, also differ in significant ways.

The simultaneous presence of the reduced HR and elevated general resistance phenotypes is perhaps not surprising, given previous evidence for feedback loops that link necrotic cell death and induction of SAR and local resistance (Dangl et al. 1996; Ryals et al. 1996). The *ihr*, *dnd*, and other mutants reported in this study may activate defense in a way that secondarily activates cell death suppression or otherwise interferes with the HR. However, it is also possible that the reduced-HR mutations first disrupt the HR and/or other cell death pathways, and secondarily activate defenses. The molecular mechanisms that underlie the phenotypes of the presently reported mutants remain to be discovered. A number of biochemical processes have been implicated in defense signaling, including the action of protein kinases, SA, reactive oxygen species, nitric oxide, and calcium-mediated signaling events (Delledonne et al. 1998; Durner et al. 1998; Hammond-Kosack and Jones 1996; Scheel 1998). Perturbation of these or other processes might account for the observed alterations in defense activation and HR. The *ihr*, *dnd*, and related mutants isolated in the present study provide an entry point for future research, and further study of these lines should reveal new information on the structure and regulation of pathogen defense and cell death pathways in plants.

MATERIALS AND METHODS

Pathogens.

P. syringae pv. *glycinea* race 4 (Psg R4) and RUU1 (another race 4 strain) were originally obtained from N. T. Keen of the University of California-Riverside. *P. syringae* pv. *tomato* (Pst) DC3000 was originally provided by D. Cuppels of the University of Ontario. The plasmids pV288, pavrRpm1, pVB01, and pKec218 carry the *avr* genes *avrRpt2*, *avrRpm1*, *avrB*, and *avrRps4*, respectively, on the plasmid pVSP61 (W. Tucker, DNA Plant Technology, Oakland, CA; Bisgrove et al. 1994; Hinsch and Staskawicz 1996; Kunkel et al. 1993). The plasmids were introduced into Psg or Pst by tri-parental mating with the helper plasmid pRK2013 (Figurski and Helinski 1979). *P. syringae* were grown at 28°C either in NYG broth (5 g of Bactopeptone, 3 g of yeast extract, and 20 ml of glycerol per liter; Daniels et al. 1984) or on NYGA plates (NYG + 1.5% agar) with kanamycin (25 µg/ml) and/or Rifampicin 50 µg/ml. Cells were resuspended to the specified density in 10 mM MgCl₂ prior to use for plant inoculation.

Spores from *P. parasitica* Noco2 were resuspended in H₂O at 4 × 10⁴ spores per ml and sprayed on 9-day-old seedlings (Parker and Coleman 1997). H₂O was used as a negative control. The inoculated seedlings were grown in a growth chamber with nearly 100% humidity at 18°C. Macroscopic phenotypes were monitored at days 3, 5, and 6.

Mutant screen.

A screening method, previously described in brief (Yu et al. 1998), was designed to allow infiltration of *P. syringae* into some but not all leaves of large numbers of plants. *Arabidopsis thaliana* ecotype Columbia (Col-0) ethylmethane-sulfonate (EMS) mutagenized M₂ seeds were obtained from Lehle Seeds (Round Rock, Texas). Plants were grown in 50-cm-long, 1.2-cm-diameter polyvinyl chloride (PVC) tubing that had been split longitudinally to leave approximately 60% of the tube circumference. Tubes were capped at the ends, drain

holes were added, and tubes were filled with potting soil (Sunshine mix #1; Sun Gro Horticulture, Bellevue, WA). Approximately 50 M₂ seeds, as well as 3 Col-0 and 3 Col-0 *rps2-201/rps2-201* seeds as controls, were planted in each tube. All seeds were stratified at 4°C for 2 days before being placed in a growth chamber. The growth chamber was set for a 9 h light/15 h dark photoperiod at 22°C. After 2 weeks, plants were transferred to continuous light for another 4 weeks. Twenty minutes before infiltration with pathogen, approximately 25 ml of 0.5% Bacto-Agar (Difco, Detroit, MI.) was added on the top of the soil to prevent loss of soil during the infiltration process. To inoculate plants, the tubes with growing plants were rotated axially 90° and set in plasticene end-mounts in a trough made of 5-cm-diameter, 60-cm-long PVC tube that had been split longitudinally, capped at the end, and filled with a solution of *P. syringae* pv. *glycinea* race 4 (Psg R4) pV288 (*avrRpt2*⁺) at a concentration of 2 × 10⁸ CFU per ml. Approximately half of the leaves on each plant were immersed in pathogen solution. Entire set-ups were then placed in a large vacuum chamber, a vacuum was drawn for about 2 min or until the solution was bubbling vigorously, and the vacuum was then released. Inoculated plants were scored visually for development of HR-associated tissue collapse 24 and 40 h after inoculation. M₂ plants that exhibited either a delayed HR or no HR on inoculated leaves were transplanted to standard pots and soil for further testing. These putative mutants were re-inoculated with Psg R4 and Psg R4 *avrRpt2*⁺ 3 weeks after being transplanted (see HR assay below). The mutants that retained the altered-HR phenotype in response to Psg R4 *avrRpt2*⁺ were saved and grown to maturity for seeds. Backcrosses of mutants to wild-type Col-0 were pursued with original M₂ plants. Progeny plants were grown in 9 h light/15 h dark at 22°C in a growth chamber for 6 weeks prior to further testing.

Genetic analysis.

Crosses were made between the putative mutants and Col-0, Col-0 *gl-1/gl-1*, an *rps2* mutant (Col-0 *rps2-201/rps2-201*), and Nössen (No-0) to perform dominance, complementation, and inheritance tests, and to reduce the presence of secondary mutations. To verify crosses, PCR was performed as in Ausubel et al. (1997) with Arabidopsis genomic DNA and primers RPS2-48-2 (5'-GCTCCTCCTAAAGTGAT), D203RPS2 (5'-TACTTGGAAAACCTAC), and RPS2-46Sal (5'-AGACGGTTCGACAAATGGATTTCATCTCA) to detect the *rps2-201C* allele (Bent et al. 1994), or nga106F (5'-GTTATGGAGTTTCTAGGGCACG) and nga106R (5'-TGCCCCATTTTGTCTTCTC) to distinguish between ecotypes (Bell and Ecker 1994). In each experiment, separate reactions were performed with genomic DNA from reference plant genotypes as positive and negative controls. Allelism between new mutations and *RPS2* was detected in F₁ plants as failure to complement the Col-0 *rps2-201/rps2-201* line for HR in response to Psg R4 *avrRpt2*⁺. Inheritance of the altered-HR phenotype and other traits was examined in F₁, F₂, and F₃ populations from the above crosses.

HR, disease, and bacterial growth assays.

HR assays were performed as previously described (Klement et al. 1964; Yu et al. 1998) with pipette or vacuum infiltration used to inoculate plants with 1 to 2 × 10⁸ CFU per

ml of Psg R4 expressing *avr* genes to induce visible leaf collapse (= HR) in incompatible interactions. All HR assays included Psg R4 pVSP61 (no *avr* gene) as a control. Data reported in Tables 1 and 2 represent cumulative values from multiple experiments. Surfactant dip disease assays used Pst strains resuspended in 0.01% Silwet L-77, 10 mM MgCl₂ as described (Whalen et al. 1991). For disease assays with syringes or pipettes, plants were inoculated with Pst DC3000 *avrRpt2*⁺ or Pst DC3000 at 5 × 10⁵ CFU per ml and disease was scored 4 days after inoculation. Bacterial growth in leaves was monitored as described following vacuum infiltration of Pst strains resuspended at 5 × 10⁴ CFU per ml (Whalen et al. 1991).

RNA blots and SA.

RNA blots were performed with inoculated and noninoculated leaf tissue samples as described (Yu et al. 1998). SA levels were determined on triplicate samples of mature leaf tissue as described (Uknes et al. 1993). Y15 lines expressing *nahG* (salicylate hydroxylase) under control of a double-35S promoter were obtained by floral dip transformation (Clough and Bent 1998) with pCIB200 (Gaffney et al. 1993); data are reported for T₃-generation plants from four independent transgenic lines homozygous for a single-locus transgene insertion.

ACKNOWLEDGMENTS

We thank Jane Parker for performing many of the *Peronospora parasitica* assays, Novartis (NABRI) for providing pCIB200 and facilities for salicylic acid studies, Thom Hoffman, J. Scott Schmidt, and Gracia Zabala for help with mutant screening and analysis, and Grace Jurkowski for critical review of the manuscript.

NOTE ADDED IN PROOF

At our new location in Wisconsin we have observed infrequent lesion mimic spotting on leaves of a small minority of Y16 (*dnd1*) and Y3 (*dnd2*) plants. Some but not all colleagues who have grown Y16 and Y3 plants at other institutions have also observed these lesions, which are apparently elicited by an unidentified environmental factor.

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