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Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance

(systemic acquired resistance/*nim* mutants/*Peronospora parasitica*/salicylic acid/2,6-dichloroisonicotinic acid)

T. P. DELANEY*, L. FRIEDRICH, AND J. A. RYALS†

Ciba–Geigy Agricultural Biotechnology, 3054 Cornwallis Road, Research Triangle Park, NC 27709-2257

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ABSTRACT **Plants possess multiple resistance mechanisms that guard against pathogen attack. Among these are inducible systems such as systemic acquired resistance (SAR). SAR is activated by pathogen exposure and leads to an increase in salicylic acid (SA), high-level expression of SAR-related genes, and resistance to a spectrum of pathogens. To identify components of the signal transduction pathways regulating SAR, a mutant screen was developed that uses 2,6-dichloroisonicotinic acid as an activator of SAR gene expression and pathogen resistance, followed by assays for resistance to the fungal pathogen *Peronospora parasitica*. Mutants from this screen were subsequently examined to assess their defense responses. We describe here a recessive mutation that causes a phenotype of insensitivity to chemical and biological inducers of SAR genes and resistance. These data indicate the existence of a common signaling pathway that couples these diverse stimuli to induction of SAR genes and resistance. Because of its *n*on-inducible *im*munity phenotype, we call this mutant *nim1*. Although *nim1* plants fail to respond to SA, they retain the ability to accumulate wild-type levels of SA, a probable endogenous signal for SAR. Further, the ability of *nim1* plants to support growth of normally incompatible races of a fungal pathogen indicates a role for this pathway in expression of genetically determined resistance, consistent with earlier findings for transgenic plants engineered to break down SA. These results suggest that the wild-type *NIM1* gene product functions in a pathway regulating acquired resistance, at a position downstream of SA accumulation and upstream of SAR gene induction and expression of resistance.**

Plant defense against disease is mediated by both pathogen-specific and general mechanisms that act against a variety of pathogens. The inducible system known as systemic acquired resistance (SAR) becomes activated by pathogen exposure and leads to a broad range of resistance against diverse pathogens. The essential role of salicylic acid (SA) for the induction of SAR was demonstrated by introducing into plants a bacterial salicylate hydroxylase gene (*nahG*) whose product converts SA to catechol. These NahG plants are unable to accumulate SA and cannot be induced for SAR (1). Interestingly, SA also appears to be required for the expression of pathogen-specific resistance: salicylate hydroxylase-expressing *Arabidopsis thaliana* plants support substantial growth of normally incompatible races of *Pseudomonas syringae* and *Peronospora parasitica* (2). The requirement for SA in both SAR and genetically determined resistance has led to the suggestion that in each of these resistance mechanisms, a common molecular pathway may function that is dependent upon SA accumulation (2). This pathway can be activated by treatment of plants with salicylic acid or 2,6-dichloroisonicotinic acid (INA). Both of these compounds induce the same set of SAR genes and the same spectrum of resistance to pathogens as found in pathogen-induced SAR (3–7). These similarities, together with the finding that salicylate-degrading (*nahG*-expressing) plants retain their ability to respond to INA, suggest that INA acts through the same pathway and at the same site or downstream from the site of SA action (2, 7).

The mechanisms by which plants couple SA perception, SAR gene induction, and manifestation of resistance are unknown. The importance of this pathway in multiple modes of disease resistance has led us to target this induction cascade for analysis. We have focused on the *A. thaliana* system for molecular and genetic analyses of disease resistance and host responses (2, 4, 8). We describe here a mutant that retains the ability to accumulate SA in response to pathogen yet has lost its ability to induce SAR genes or resistance after application of SA or INA. Because of its *n*on-inducible *im*munity phenotype, we call this mutant *nim1*. The inability of *nim1* plants to respond to chemical inducers of resistance is accompanied by decreased pathogen inducibility of SAR genes. Further, *nim1* plants support growth of normally incompatible races of a fungal pathogen, suggesting a defect in genetically determined resistance, similar to that found in plants engineered to degrade SA (2).

MATERIALS AND METHODS

Plant Lines and Fungal Strains. *A. thaliana* ecotype Wassilewskija (Ws-O; stock number CS 2360) and fourth-generation (T_4) seeds from T-DNA-transformed lines (9) were obtained from the Ohio State University *Arabidopsis* Biological Resource Center (Columbus). Second-generation (M_2) seeds from ethyl methanesulfonate-mutagenized Ws-O plants were obtained from Lehle Seeds (Round Rock, TX). SA quantitation experiments employed *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 containing the cloned *avrRpt2* gene [DC3000(*avrRpt2*)], obtained from B. Staskawicz, University of California, Berkeley (10). *P. parasitica* pathovars and their sources were as follows: Emwa (11) from E. Holub and I.R. Crute, Horticultural Research Station, East Malling, Kent; Wela (12) from A. Slusarenko and B. Mauch-Mani, Institut für Pflanzenbiologie, Zürich, Switzerland; and Noco (13) from J. Parker, Sainsbury Laboratory, Norwich, England. Fungal cultures were maintained by weekly culturing (4) on *Arabidopsis* ecotype Ws-O, Weiningen, and Col-O, for *P. parasitica* pathovars Emwa, Wela, and Noco, respectively.

Mutant Screen. M_2 or T_4 seeds were grown on soil for 2 weeks under 14 hr of light per day, misted with 0.33 mM INA (0.25 mg/ml, made from 25% INA in wettable powder; Ciba, Basel), and inoculated 4 days later by spraying a *P. parasitica* conidial suspension containing 5–10 × 10^4 conidiospores per ml of water. Plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation. Potential

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Abbreviations: INA, 2,6-dichloroisonicotinic acid; SA, salicylic acid; SAR, systemic acquired resistance; *Pst*, *Pseudomonas syringae* pv. *tomato*.

*Present address: Department of Plant Pathology, Cornell University, 334 Plant Science Building, Ithaca, NY 14853.
†To whom reprint requests should be addressed.

mutant lines were transferred to warmer and drier conditions to produce seed, which was retested by the same screen.

Genetic Analyses. Pollen from kanamycin-resistant mutant plants was transferred to stigmata of Ws-O (wild-type) plants. Seeds resulting from the cross were plated onto Murashige–Skoog B5 plates (14) containing kanamycin at 25 μg/ml to verify the hybrid origin of the seed. Twenty-four kanamycin-resistant (F_1) plants were transferred to soil and assayed for mutant phenotype by INA induction of *P. parasitica* pv. Emwa resistance. F_2 seeds from these plants were grown on soil, and 130 randomly selected F_2 plants were examined for segregation of the mutant phenotype by the pathogen assay described above.

RNA Blot Analyses. RNA prepared from treated and control plants was size-fractionated by agarose gel electrophoresis and transferred to GeneScreen*Plus* membranes (DuPont). Replicate blots were hybridized to *Arabidopsis* SAR gene probes PR-1, PR-2, and PR-5 as described (6). Loadings were standardized by measuring UV absorbance of RNA samples, ethidium bromide staining of gels, and hybridization to a β-tubulin ABT-4 gene probe (PCR amplified from *Arabidopsis* DNA with primers spanning positions 1050–2150; GenBank accession no. M21415) (15).

Fungal Growth. *In planta* fungal growth was examined by lactophenol trypan blue staining (4).

Determination of SA Levels in Plants. Wild-type and mutant plants were grown for 4 weeks. Several leaves from each plant were infiltrated by a syringe with *Pst* DC3000(*avrRpt2*) (5×10^6 bacteria per ml in 10 mM $MgCl_2$) or with 10 mM $MgCl_2$ alone. Leaf samples were collected from plants before infiltration and 2 days after infiltration. This time point was selected because pilot experiments had shown SAR genes to be induced within 2 days of DC3000(*avrRpt2*) treatment; at later times, pathogen-caused tissue destruction prevented sampling infiltrated leaves (unpublished results). Samples were collected from DC3000(*avrRpt2*)-treated plants and separated into primary-infiltrated and secondary noninfiltrated leaf samples. $MgCl_2$ (vehicle)-treated samples were collected as negative controls. Three independent 0.4-g (fresh weight) samples were prepared from each treatment. Both free SA and total SA following hydrolysis with β-glucosidase were quantified by HPLC. Extraction efficiency ranged from 29% to 67% and was determined from the recovery of added SA to several noninduced samples. SA extraction and HPLC methods were as described (4).

RESULTS

Isolation of *nim1* Plants. *Arabidopsis* plants treated with INA show complete resistance to the oomycete *P. parasitica*, which causes downy mildew disease (Fig. 1 *A* and *C*). Screening

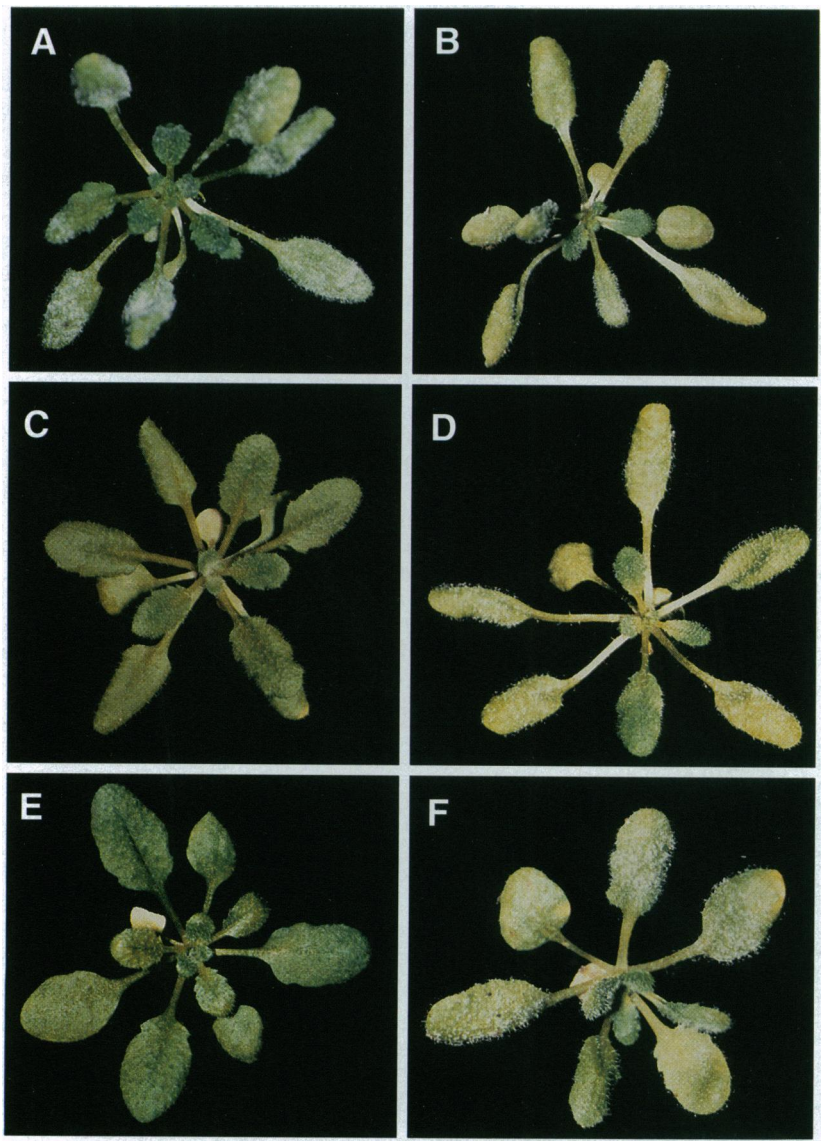

FIG. 1. *nim1* plants are defective in acquired resistance to *P. parasitica* following treatment with SA or INA. Wild-type (*A*, *C*, and *E*) and *nim1* (*B*, *D*, and *F*) plants were treated with water (*A* and *B*), INA (*C* and *D*), or SA (*E* and *F*). Four days later, plants were inoculated with a *P. parasitica* pv. Emwa conidial suspension. Evaluations and photographs were made 7 days later. After each treatment, fungal growth on *nim1* was indistinguishable from that on water-treated wild-type plants.

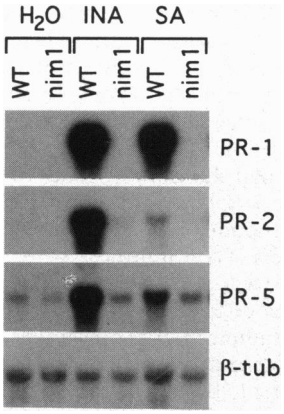

FIG. 2. Chemical induction of SAR genes is diminished in *nim1* plants. Water, INA, or SA was applied to wild-type (WT) and *nim1* plants. After 4 days, RNA was prepared from these plants and examined for expression of PR-1, PR-2, PR-5, and β-tubulin (loading control).

of 280,000 M_2 plants (from ethyl methanesulfonate mutagenesis) and 80,000 T_4 plants (from T-DNA transformation) identified 75 lines that supported high levels of *P. parasitica* pv. Emwa growth despite pretreatment with INA. Progeny testing identified 6 lines that yielded 100% INA-insensitive plants (Fig. 1 *B* and *D*). Line T-17, from one of the T-DNA-transformed populations, was the first isolated and is characterized here. The additional *nim* lines were more recently identified; their phenotypic and genetic analyses will be presented elsewhere. Because the screen could have identified mutant phenotypes affected only in INA action, we asked whether SA would also fail to function in the INA-insensitive mutants. In contrast to its ability in wild-type plants, SA did not induce resistance in the T-17 mutant line (Fig. 1 *E* and *F*). Because both INA and SA failed to induce resistance in the T-17 line, we call this line *nim1*, for its *n*on-inducible *im*munity phenotype.

SA and INA Treatment Fails to Induce SAR Genes in *nim1* Plants. A hallmark of SAR is the induction of a broad set of genes, some of which have been shown to confer resistance to specific pathogens (3, 16, 17). The association of pathogen resistance with SAR gene expression is also supported by the finding that several *Arabidopsis* mutants constitutively express SAR genes and manifest enhanced resistance to pathogens (8). Furthermore, resistance induced by INA or SA treatment is always accompanied by accumulation of SAR gene products. To determine whether the inability of *nim1* plants to develop resistance following INA or SA treatment was associated with altered SAR gene induction, we tested the ability of these chemicals to induce SAR genes in *nim1* plants. PR-1, PR-2, and PR-5 induction by INA and SA was substantially reduced in *nim1* plants compared with wild-type plants (Fig. 2).

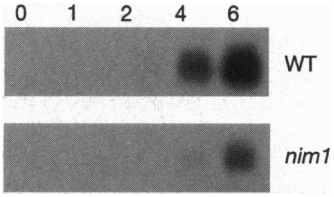

FIG. 3. Pathogen induction of PR-1 is diminished in *nim1* plants. Wild-type (WT) and *nim1* plants were spray-inoculated with *P. parasitica* pv. Emwa. Samples were collected at days 0, 1, 2, 4, and 6 and RNA was analyzed by blot hybridization with an *A. thaliana* PR-1 cDNA probe to measure PR-1 mRNA accumulation.

Pathogen Induction of PR-1 Is Reduced in *nim1* Plants. *nim1* plants exhibit dual insensitivity to two chemical effectors of acquired disease resistance, INA and SA, in both pathogen susceptibility and gene expression assays. Because SAR-associated genes are induced by pathogen exposure in a SA-dependent-manner (1, 2), we predicted that *nim1* plants would be defective in pathogen induction of SAR genes. Therefore, we compared *nim1* and wild-type plants for induction of PR-1 following infection with *P. parasitica* pv. Emwa. Over the course of a 6-day infection cycle, PR-1 mRNA accumulation was lower in *nim1* plants than in wild-type plants (Fig. 3).

SA Accumulates Following Pathogen Infection. The phenotype of *nim1* plants suggested a disruption in a component of the signaling pathway downstream of SA and upstream of SAR gene induction. We therefore predicted that *nim1* plants would accumulate SA following pathogen infection as do wild-type plants. After induction by *Pst*, the levels of free and total SA were at least as high in *nim1* as in wild-type plants (Fig. 4). Similar results were observed in both primary (inoculated) and secondary (noninoculated) leaves, although induction of SA was much less in secondary than in primary leaves in both wild-type and *nim1* plants (Fig. 4). Previous experiments in tobacco following tobacco mosaic virus infection have shown that secondary leaves in these plants also accumulate less SA than primary infected leaves (18, 19).

Incompatible Fungal Pathogens Cause Disease on *nim1* Plants. Experiments with SA-degrading plants have shown that SA depletion compromised the plants' ability to manifest genetically determined resistance (2). Because the *nim1* mutation interferes with plant responses to SA, we asked whether these plants might also support growth of incompatible pathogens. Several *P. parasitica* isolates differing in their host ranges were tested. After infection with the compatible fungal pathovar Emwa, *nim1* plants showed (Fig. 5 *A* and *B*) disease symptoms and progression similar to those observed on wild-type Ws-O plants (Fig. 1 *A* and *B*; unpublished results). The Wela and Noco pathovars of *P. parasitica* are unable to grow

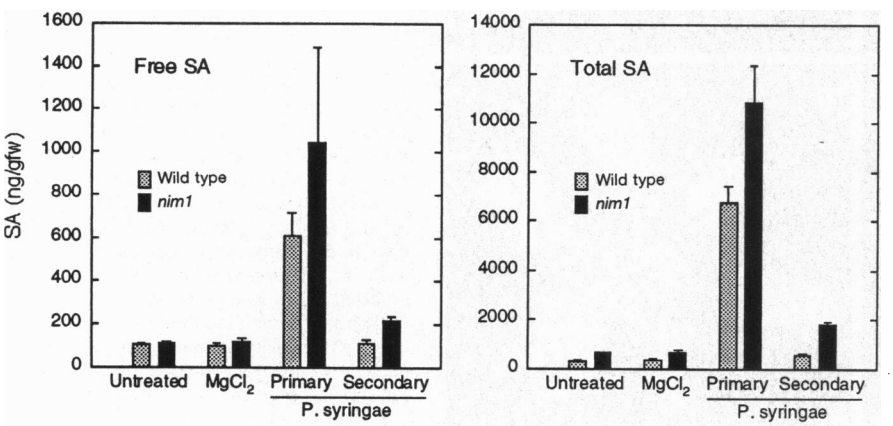

FIG. 4. *nim1* plants accumulate SA following pathogen exposure. Leaves of wild-type and *nim1* plants were infiltrated with *Pst* DC3000(*avrRpt2*) or carrier medium (10 mM $MgCl_2$) alone. After 2 days, samples were collected from untreated, $MgCl_2$-treated, and DC3000(*avrRpt2*)-treated plants. Bacteria-treated samples were separated into primary infiltrated and secondary (noninfiltrated) leaves. Free SA (*Left*) and total SA following hydrolysis with β-glucosidase (*Right*) were quantified by HPLC. Error bars indicate SD of three replicate samples.

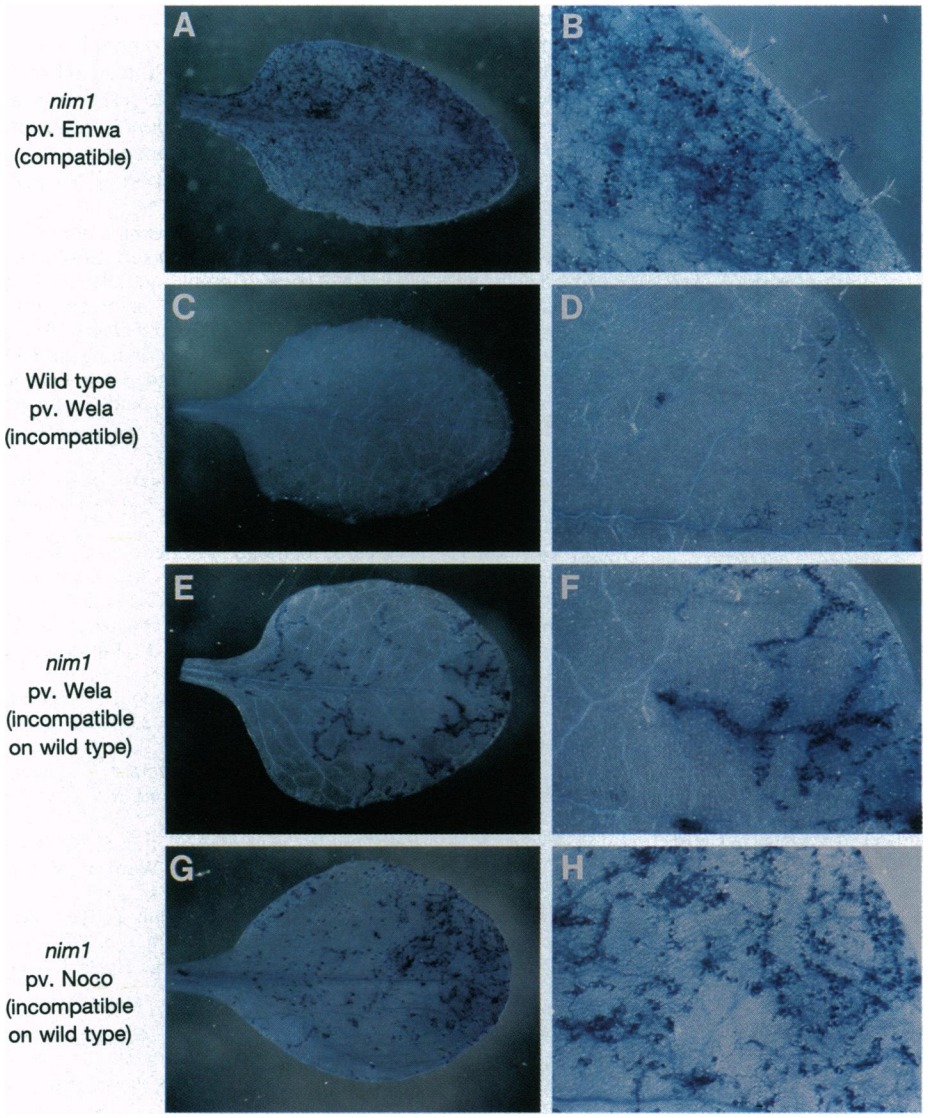

FIG. 5. *nim1* plants are defective in expression of genetically determined resistance. Infection of wild-type (Ws-O) or *nim1* plants with the compatible pathogen *P. parasitica* pv. Emwa leads to profuse fungal development (*nim1* shown in *A* and *B*). Infection of wild-type plants with the incompatible *P. parasitica* pv. Wela does not lead to disease (*C* and *D*). However, the otherwise isogenic *nim1* mutant does support growth of this normally incompatible pathovar (*E* and *F*). Tests of *P. parasitica* pv. Noco, which is incompatible on Ws-O (data not shown), show that *nim1* plants also support limited growth of this fungal isolate (*G* and *H*).

on the *Arabidopsis* Ws-O ecotype, due to the presence of host resistance genes that recognize avirulence determinants in these fungi (Wela shown, Fig. 5 *C* and *D*) (11, 13). However, when Wela or Noco was inoculated onto *nim1* plants, a degree of compatibility was indicated by hyphal development. Growth of the normally incompatible Wela isolate in *nim1* plants (Fig. 5*E*) was accompanied by a host reaction visible at higher magnification (Fig. 5*F*), in the form of necrotic plant cells along the hyphal filaments. Infection of *nim1* plants with Noco also led to disease symptoms qualitatively different from those of wild-type Ws-O plants infected with this fungal isolate (Fig. 5 *G* and *H*; unpublished data). While *nim1* plants demonstrated relaxed control of normally incompatible fungal pathogens, the growth of these agents was not as profuse as on their normal wild-type hosts.

Genetic Analysis. To determine the genetic characteristics of the *nim1* allele, mutant plants were backcrossed to the wild type. In the F_1 progeny of this cross, normal INA responsiveness was observed in pathogen assays following INA treatment (24 out of 24 F_1 plants) (Table 1). Several F_2 progeny groups derived from these F_1 plants were examined for segregation of the *nim1* mutant phenotype by the same assay (130 F_2 plants were examined). In each progeny group, a 3:1 ratio of wild-type to *nim1* mutant phenotype was observed (Table 1). Together, these data indicate that the *nim1* allele isolated is recessive and identifies a single genetic locus.

DISCUSSION

To gain access to regulatory pathway components that govern acquired resistance, a mutant screen was developed that utilizes chemical induction of resistance followed by challenge with a fungal pathogen. With this approach, we identified a mutant line called *nim1* that fails to respond to both biological and chemical effectors of resistance. In wild-type plants, the *NIM1* gene appears to encode an essential component of the signal transduction pathway controlling SAR gene expression and resistance. This idea is supported by several observations: (*i*) Unlike wild-type plants, in which SAR genes are highly induced by either synthetic (INA) or endogenous (SA) chemicals or by pathogen exposure, *nim1* plants simultaneously lose response to all of these agents. (*ii*) The loss of gene induction affects several SAR genes coordinately, consistent with a defect in a regulatory gene product. (*iii*) After INA or SA treatment, the defective gene-induction

Table 1. *nim1* × Ws-O cross shows that the *nim1* phenotype is due to a recessive mutation in a single gene

Generation	No. of plants with phenotype: Wild-type	*nim1*	χ^2 (3:1)
F_1	24	0	
F_2	98	32	$0.95 < P < 0.9$

phenotype is associated with a lack of resistance to a compatible isolate of *P. parasitica*. (*iv*) *nim1* plants support growth of normally incompatible strains of *P. parasitica*, similar to the phenotype of SA-degrading plants (2). Therefore the *nim1* mutation leads to an inability to respond to diverse stimuli that normally lead to induction of a spectrum of SAR genes and resistance.

By design, the mutant screen used in this study targeted a specific part of the pathway governing resistance, delimited by the site of action of INA, and the induction of SAR genes and resistance. To define the upstream limit of action for the *NIM1* gene product, we measured SA levels in *nim1* plants. We found that *nim1* plants accumulated at least as much SA as wild-type plants following pathogenesis. Therefore, we believe that the mutant phenotype is not due to errors in SA regulation or production, but rather to defects in either SA perception or events subsequent to SA sensing. Interestingly, SA levels following pathogenesis appear elevated in *nim1* plants compared with wild type. To determine whether the *NIM1* pathway plays a role in modulating SA levels will require further investigation.

Using a mutant screen based on the PR-2 promoter-driven expression of the β-glucuronidase reporter gene, Cao *et al.* (20) recently isolated and described an *Arabidopsis* mutant called *npr1*, with reduced SA and INA inducibility of SAR genes and resistance to bacterial pathogens. *npr1* plants were not characterized with respect to SA accumulation or induction of SAR genes in response to pathogen, making further comparisons to *nim1* difficult at this time.

Our data indicate the existence of a common signaling pathway, identified by the *nim1* mutation, that couples pathogen, SA, and INA perception with induction of SAR genes and resistance. These signals converge either at or upstream of the site of NIM1 function. Furthermore, the INA-nonresponsive phenotype of *nim1* plants demonstrates that the mode of action of INA is through activation of authentic plant resistance mechanisms that are also utilized following pathogen perception or SA accumulation. A corollary of this conclusion is that INA itself is not directly fungicidal, consistent with data showing that INA does not interfere with fungal growth *in vitro* (21).

Despite the combined insensitivity to INA and SA exhibited by *nim1* plants, they were no more susceptible to the compatible Emwa pathovar of *P. parasitica* than wild-type plants. This is in contrast to results found in NahG plants, where SA depletion caused enhanced susceptibility to compatible pathogens (2). The greater pathogen susceptibility found in NahG compared with *nim1* plants may have several explanations. (*a*) Because NahG plants are from the Col-0 ecotype, whereas *nim1* plants are ecotype Ws-O, we used different pathovars of *P. parasitica* in evaluating these plants. If the virulence properties differed in these fungal strains, one might observe differences such as those described. (*b*) It is possible that expression of salicylate hydroxylase disables the NIM1 pathway more completely than the *nim1* mutant allele described, which would in this case be considered a "leaky" allele. However, we do not have evidence for partial function of the *NIM1* gene product in *nim1* plants. Finally, the possibility exists that separate SA-dependent pathways function to limit disease progression. In this case, one of the SA-dependent pathways is defined by the *nim1* mutation, while another, NIM1-independent pathway is partially responsible for the phenotype of NahG plants. Consequently, SA depletion in NahG plants would simultaneously disable both the NIM1 pathway and another pathway, leading to hypersusceptibility.

Interestingly, *nim1* and SA-depleted plants share the ability to support limited growth of normally incompatible *P. parasitica* strains. This observation suggests that the NIM1 pathway is necessary for the manifestation of genetic resistance, which is inactivated by either SA depletion or the *nim1* mutation. We therefore propose a role in genetic resistance for SAR gene expression or other processes dependent upon NIM1 activity.

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