

# Interaction of NIMIN1 with NPR1 Modulates *PR* Gene Expression in Arabidopsis

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**The *Arabidopsis thaliana* NONEXPRESSER OF *PR* GENES1 (NPR1, also known as NIM1) protein is an essential positive regulator of salicylic acid (SA)-induced *PATHOGENESIS-RELATED (PR)* gene expression and systemic acquired resistance (SAR). *PR* gene activity is regulated at the level of redox-dependent nuclear transport of NPR1. NPR1 interacts with members of the TGA family of transcription factors that are known to bind to SA-responsive elements in the *PR-1* promoter. In an attempt to identify proteins involved in SA-mediated signal transduction, we previously described the isolation of three novel genes encoding distinct albeit structurally related proteins designated NIMIN1 (for NIM1-INTERACTING1), NIMIN2, and NIMIN3 that interact with NPR1 in the yeast two-hybrid system. Here, we show that NIMIN1 and NPR1 can be copurified from plant extracts, providing biochemical evidence for their interaction. We provide functional evidence for this interaction by describing transgenic plants constitutively expressing high amounts of NIMIN1. These plants show reduced SA-mediated *PR* gene induction and a compromised SAR, thus mimicking the described phenotype conferred by *npr1*. Moreover, they showed reduced *RESISTANCE* gene-mediated protection. These effects were dependent on the ability of NIMIN1 to interact with NPR1. Mutant plants with a T-DNA insertion in *NIMIN1* as well as transgenic plants with reduced *NIMIN1* mRNA levels showed hyperactivation of *PR-1* gene expression after SA treatment but no effect on the disease resistance phenotype. Our results strongly suggest that NIMIN1 negatively regulates distinct functions of NPR1, providing a mechanism to modulate specific features of SAR.**

## INTRODUCTION

Nutrients synthesized by plants attract all kinds of organisms. To avoid being exploited, plants have evolved sophisticated mechanisms to protect themselves against pathogens. These include both preformed and pathogen-inducible physical and chemical barriers. *RESISTANCE (R)* gene-mediated protection, for example, is an induced response. A so-called avirulence (*Avr*) gene product of the pathogen is recognized directly or indirectly by a plant *R* gene product (for recent reviews, see Belkadir et al., 2004; Innes, 2004). *R* gene-mediated recognition triggers a diverse set of cellular responses. Signaling molecules such as reactive oxygen species, nitric oxide, jasmonic acid (JA), ethylene, and salicylic acid (SA) are produced, and defense-related genes (e.g., the *PATHOGENESIS-RELATED [PR]* genes) are transcriptionally activated (Lamb and Dixon, 1997). This often coincides with the formation of necrotic lesions at the site of

infection, which is termed the hypersensitive response. The pathogen is contained in these lesions and thus unable to spread throughout the plant (Dangl et al., 1996). Ultimately, the plant develops a kind of long-lasting, systemic immunity to subsequent challenge by a wide range of normally virulent pathogens. This immunity is called systemic acquired resistance (SAR) (Uknes et al., 1992; Ryals et al., 1994). SAR is preceded by an accumulation of SA, which is observed not only locally during the hypersensitive response but subsequently also in distal uninfected tissue. This leads to the concerted induction of a subset of *PR* genes that code for proteins such as  $\beta$ -glucanases and chitinases (Van Loon and Van Kammen, 1970; Ward et al., 1991). *PR* genes can also be induced by exogenous application of SA (White, 1979). They are commonly used as marker genes for SAR. SA is a necessary and sufficient signal for SAR, because *Arabidopsis thaliana* mutants defective in SA biosynthesis are strongly impaired in SAR (Wildermuth et al., 2001).

A plethora of genetic screens has been conducted to identify proteins involved in the signal transduction leading to SAR. Several groups have identified mutants in the *NONEXPRESSER OF PR GENES1 (NPR1)*, also known as *NIM1* and *SAI1* locus, which are impaired in SA-induced *PR* gene expression and SAR (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). The analysis of *npr1* mutants and the fact that lines overexpressing NPR1 show enhanced disease resistance led to the conclusion that NPR1 is a key positive regulator of SAR (Cao et al., 1997, 1998). The question of how NPR1 acts as

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a mediator between the SA signal and the activation of *PR* gene expression was investigated intensively during the last years. It was recently demonstrated that *PR* gene activity is regulated at the level of redox-dependent nuclear localization of NPR1 (Kinkema et al., 2000; Mou et al., 2003). Before SAR induction, NPR1 resides in a homooligomeric complex in the cytoplasm. After induction of SAR, SA accumulation triggers a redox change that brings about monomerization of NPR1, presumably as a result of the reduction of intermolecular disulfide bridges. In its monomeric form, NPR1 is transported to the nucleus and activates *PR* gene expression. The mechanism by which NPR1 regulates *PR* gene activity is of great interest. Analysis of the NPR1 primary structure predicted neither a DNA binding domain nor a transcriptional activation domain (Cao et al., 1997). Thus, it is unlikely that NPR1 acts as a transcription factor itself. On the other hand, two putative protein–protein interaction domains, an ankyrin repeat domain and a BTB/POZ (for *BROAD-COMPLEX*, *TRAMTRACK*, AND *BRIC-A-BRAC*/POXVIRUS, ZINC FINGER) domain, were identified in NPR1 (Cao et al., 1997; Aravind and Koonin, 1999).

Using the yeast two-hybrid system with NPR1 as a bait, two classes of interacting proteins were identified. Several groups discovered members of the basic domain/leucine zipper family of transcription factors, the TGA factors that were demonstrated to bind to SA-responsive elements of the *PR-1* promoter in vitro (Strompen et al., 1998; Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000). The in vivo interaction between TGA2 and NPR1 was shown to be SA-dependent (Subramaniam et al., 2001). Moreover, NPR1- and SA-dependent recruitment of a TGA factor to its target sequence of the *PR-1* promoter was reported (Fan and Dong, 2002; Johnson et al., 2003). In an attempt to identify proteins involved in SA signal transduction, we previously described the isolation of three novel genes encoding distinct albeit structurally related proteins designated NIMIN1 (for NIM1-INTERACTING1), NIMIN2, and NIMIN3 that interact with NPR1 (also known as NIM1) in the yeast two-hybrid system (Weigel et al., 2001). The NIMIN proteins fail to bind a mutant NPR1 that abolishes SAR in Arabidopsis. In addition, their mRNAs are transiently expressed after SA induction. These data, together with the finding that NIMIN proteins, NPR1, and TGA factors form a ternary complex in yeast, suggest that they are involved in the signal transduction pathway leading to SAR.

Here, we show that NPR1 interacts with NIMIN1 in plant extracts and that a ternary complex of NIMIN1, NPR1, and TGA factor can bind to an SA-responsive promoter element in the yeast one-hybrid system. Analysis of plants overexpressing NIMIN1 revealed that SA-mediated *PR* gene induction is repressed and that these plants are impaired in SAR and *R* gene-mediated resistance. We demonstrate that this effect is contingent on the ability of NIMIN1 to interact with NPR1. In a complementary approach, we identified a *NIMIN1* knockout mutant and created double-stranded RNA interference (dsRNAi) transgenic lines. Both strategies yielded plants showing enhanced *PR-1* gene induction after SA treatment. However, as this hyperactivation of gene expression did not coincide with enhanced resistance, endogenous NIMIN1 seems to regulate only distinct functions of NPR1.

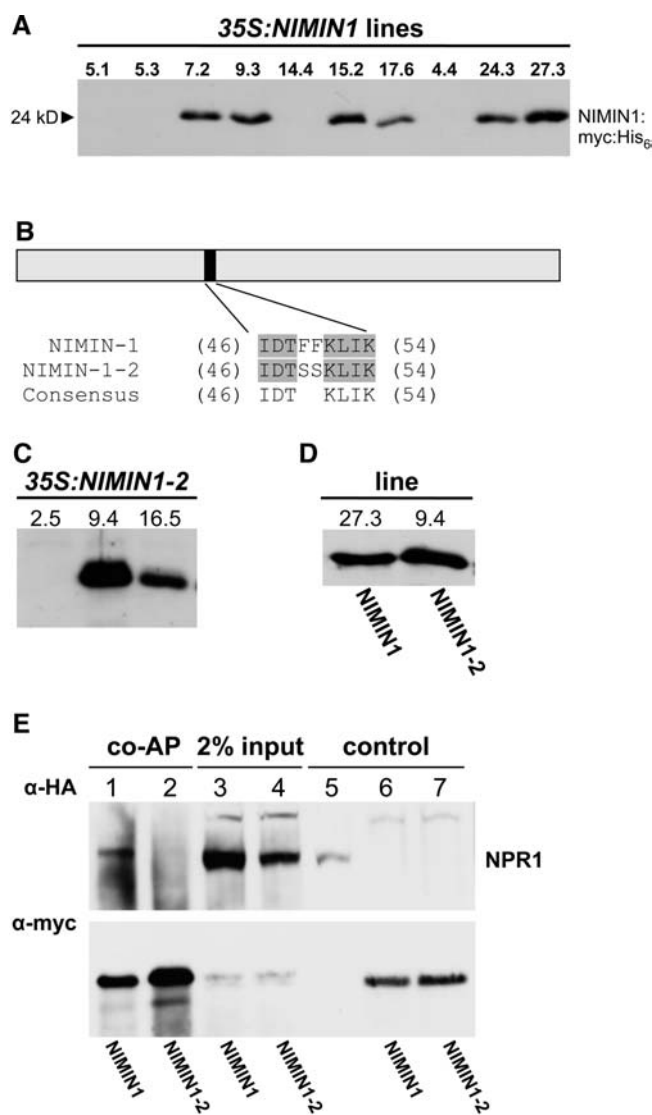
## RESULTS

### NPR1 Interacts with NIMIN1 but Not with a Mutant Derivative, NIMIN1-2, in Plant Extracts

In an attempt to identify proteins involved in SA signal transduction, we previously described the isolation of three novel genes encoding distinct albeit structurally related proteins, NIMIN1, NIMIN2, and NIMIN3, that interact with NPR1 in the yeast two-hybrid system. NIMIN2 and NIMIN3 were also shown to bind NPR1 in vitro (Weigel et al., 2001). To analyze whether this interaction occurs in planta, we created transgenic lines expressing *NIMIN1* constitutively. To facilitate the detection of NIMIN1, a sequence encoding a c-myc:hexahistidine (His<sub>6</sub>) tag was added to the C terminus of the protein. Yeast three-hybrid analysis confirmed that the NIMIN1:myc:His<sub>6</sub> fusion still forms a ternary complex with NPR1 and TGA factors (data not shown), as described previously (Weigel et al., 2001). The NIMIN1:myc:His<sub>6</sub> gene fusion under the control of the *Cauliflower mosaic virus* 35S promoter was transformed via *Agrobacterium tumefaciens*-mediated gene transfer into wild-type plants (*Arabidopsis* ecotype Columbia [Col-0]). Thirty-two transformants were selected and 10 T0 lines were brought to homozygosity (35S:*NIMIN1* lines), as inferred from segregation analysis. As shown by the protein gel blot in Figure 1A, NIMIN1:myc:His<sub>6</sub> accumulates to high levels in lines 7.2, 9.3, 15.2, 24.3, and 27.3, to intermediate levels in line 17.6, and is hardly detectable in lines 5.1, 5.3, 14.4, and 4.4. The protein migrates at a position corresponding to 24 kD, which is close to its predicted molecular mass of 21 kD. The lines with strong NIMIN1:myc:His<sub>6</sub> expression display subtle morphological changes: the petioles tend to be longer and the laminae are a bit shorter than in wild-type plants, but rosette size is similar to that of wild-type plants. They also appear to be earlier flowering than wild-type plants (data not shown).

As a control for the in planta interaction assay, we created transgenic lines expressing a NIMIN1 mutant that does not interact with NPR1. In our previous study, we had mapped the NPR1 interaction domain of NIMIN1 to amino acids 49 to 54 (Weigel et al., 2001). To identify crucial residues within the six-amino acid region that are important for the interaction with NPR1, a rational site-directed mutagenesis approach was taken based on a multiple alignment of the region of three NIMIN proteins (NIMIN1, NIMIN1b, and NIMIN2) (Weigel et al., 2001) and G8-1, a NIMIN-like protein from tobacco (*Nicotiana tabacum*) (Horvath et al., 1998). Only two consecutive Phe residues are conserved in all four proteins (Phe-49 and Phe-50 in NIMIN1). We have chosen to exchange these large hydrophobic residues by small, slightly polar Ser residues (Betts and Russell, 2003) to disrupt the NPR1 interaction domain. The NIMIN1 mutant was designated NIMIN1-2 (Figure 1B). Using the yeast two-hybrid system, we confirmed that NIMIN1-2 does not interact with NPR1 (data not shown).

To create transgenic lines expressing *NIMIN1-2*, the cDNA was fused as the wild-type cDNA to a c-myc:His<sub>6</sub> tag and was expressed under the control of the *Cauliflower mosaic virus* 35S promoter. Several lines were brought to homozygosity (35S:*NIMIN1-2* lines), as inferred from segregation analysis. As shown by the protein gel blot in Figure 1C, NIMIN1-2:myc:His<sub>6</sub>



**Figure 1.** NPR1 Interacts with NIMIN1 but Not with a Mutant Derivative, NIMIN1-2, in Plant Extracts.

**(A)** Characterization of plants overexpressing NIMIN1:myc:His<sub>6</sub> (35S:NIMIN1 lines). Protein gel blot analysis of the NIMIN1:myc:His<sub>6</sub> protein in 35S:NIMIN1 transformants. Expression levels of 10 homozygous lines are shown. NIMIN1:myc:His<sub>6</sub> was enriched from denaturing extracts using nickel affinity chromatography and detected using an antibody against the c-myc tag. The position and molecular mass of NIMIN1:myc:His<sub>6</sub> are indicated by an arrowhead at right.

**(B)** Top, scheme of NIMIN1 and its NPR1 interaction domain. Bottom, alignment of the amino acid sequences of wild-type and mutant NIMIN1.

**(C)** Characterization of plants overexpressing a mutant derivative of NIMIN1 designated NIMIN1-2 that does not interact with NPR1 (35S:NIMIN1-2 lines). Protein gel blot analysis of the NIMIN1-2:myc:His<sub>6</sub> protein in 35S:NIMIN1-2 transformants. Expression levels of three homozygous lines are shown. NIMIN1-2:myc:His<sub>6</sub> was detected using an antibody against the c-myc tag.

**(D)** NIMIN1:myc:His<sub>6</sub> and NIMIN1-2:myc:His<sub>6</sub> are expressed at similar levels in transgenic lines. Protein gel blot analysis of 35S:NIMIN1 and 35S:NIMIN1-2 transgenic lines. Native protein extracts were prepared

accumulates to a high level in line 9.4, to an intermediate level in line 16.5, and is hardly detectable in line 2.5. The subtle growth phenotype of 35S:NIMIN1 lines was also observed with 35S:NIMIN1-2 lines.

To test for interaction between NIMIN1 and NPR1 in planta, we crossed 35S:NIMIN1 line 27.3 and 35S:NIMIN1-2 line 9.4 to homozygous plants expressing a triple hemagglutinin epitope (HA<sub>3</sub>)-tagged NPR1 (35S:NPR1) in the *npr1-1* background. 35S:NIMIN1-2 line 9.4 was chosen as a control because it exhibits similar levels of transgene expression as 35S:NIMIN1 line 27.3 (Figure 1D). The resulting F1 generation was analyzed for complex formation between either NIMIN1 and NPR1 or NIMIN1-2 and NPR1 using coaffinity purification. Because NIMIN1 is localized to the nucleus (Weigel et al., 2001), nuclear localization of NPR1 was induced by SA treatment of F1 plants 8 h before harvesting. Total protein extracts of plants expressing similar levels of either NIMIN1:myc:His<sub>6</sub> and HA<sub>3</sub>:NPR1 or NIMIN1-2:myc:His<sub>6</sub> and HA<sub>3</sub>:NPR1 were prepared and incubated with nickel-nitrilotriacetic acid agarose (Ni-NTA) as described previously (Fan and Dong, 2002). His<sub>6</sub>-tagged NIMIN1 and NIMIN1-2, together with corresponding interaction partners, were bound to the matrix and were eluted after washing away nonspecific interactors. Protein gel blot analysis confirmed that similar amounts of NIMIN1 and NIMIN1-2, and similar amounts of NPR1, were present in the input extracts of both transgenic lines (Figure 1E, lanes 3 and 4, bottom and top panels), and similar amounts of either NIMIN1 or NIMIN1-2 were purified from these extracts (Figure 1E, lanes 1 and 2, bottom panel). As shown in Figure 1E, NPR1 was copurified with NIMIN1 (lane 1, top panel) but not with NIMIN1-2 (lane 2, top panel). This finding clearly demonstrates that interaction between NIMIN1 and NPR1 occurs in plant extracts and that interaction between NIMIN1-2 and NPR1 is disrupted not only in yeast but also in plant extracts.

#### A Ternary Complex of NIMIN1, NPR1, and TGA Factor Binds to the *activation sequence-1* Element in Yeast

Next, we addressed the question of whether NIMIN1 has the potential to modulate *PR* gene expression. Yeast three-hybrid data suggest that NIMIN1 may be involved in *PR* gene regulation as part of a ternary complex with NPR1 and TGA factors. In vitro experiments failed to show that NPR1 interacts with a TGA factor bound to an *activation sequence-1* (*as-1*)-like *cis* element of the *PR-1* gene promoter (Després et al., 2000). To examine whether NPR1 can interact with DNA-bound TGA factors in an in vivo

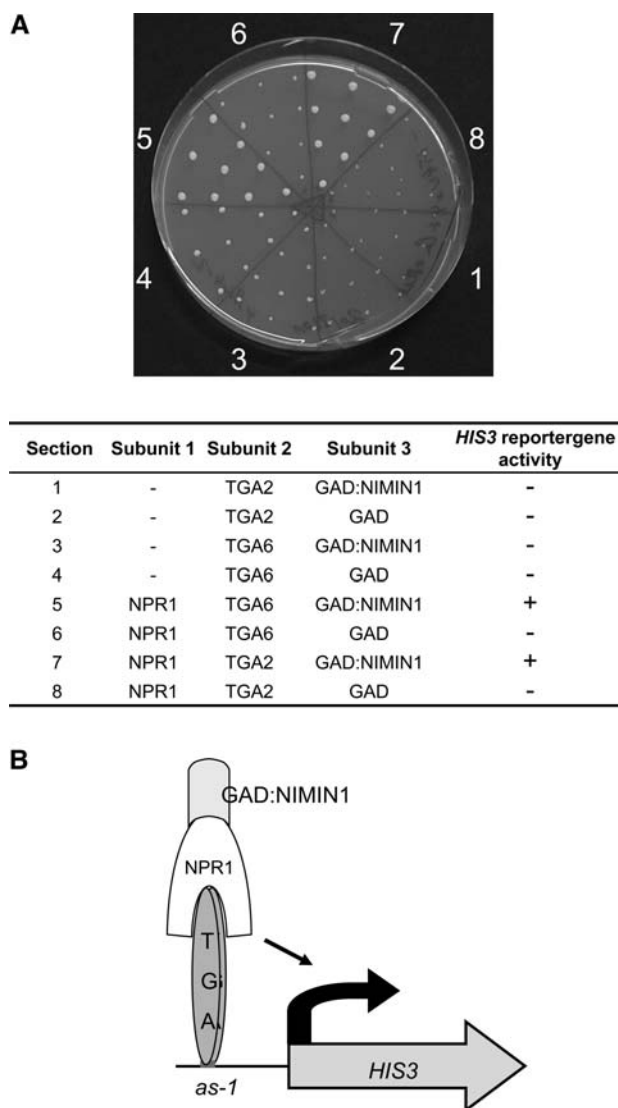
from lines strongly expressing the wild type (line 27.3) and the mutant allele (line 9.4) of NIMIN1. Total protein (50 μg) was loaded in each lane. **(E)** Coaffinity purification (co-AP) of HA<sub>3</sub>:NPR1 with NIMIN1:myc:His<sub>6</sub>. HA<sub>3</sub>:NPR1 was pulled down with NIMIN1:myc:His<sub>6</sub> (lane 1) but not with NIMIN1-2:myc:His<sub>6</sub> (lane 2) in protein extracts from transgenic plants. Top, Detection of HA<sub>3</sub>:NPR1 using an HA tag antibody. Bottom, detection of NIMIN1:myc:His<sub>6</sub> or NIMIN1-2:myc:His<sub>6</sub> using a c-myc tag antibody. Total extract (2.5 mg) from plants expressing either HA<sub>3</sub>:NPR1 and NIMIN1:myc:His<sub>6</sub> (lane 3) or HA<sub>3</sub>:NPR1 and NIMIN1-2:myc:His<sub>6</sub> (lane 4) was used for coaffinity purification with Ni-NTA. Lanes 5 to 7 show extracts from plants expressing HA<sub>3</sub>:NPR1, NIMIN1:myc:His<sub>6</sub>, or NIMIN1-2:myc:His<sub>6</sub> alone.

situation and to test whether NIMIN1 may be part of this complex, we used the yeast one-hybrid system to modulate *PR* gene expression. A yeast strain was created carrying the *HIS3* reporter gene driven by a minimal promoter with three tandemly repeated *as-1* DNA binding sites. NIMIN1 was fused to the GAL4 transcription activation domain (GAD:NIMIN1) because none of the subunits of the putative complex exhibits transcriptional activation in yeast (R. Weigel, unpublished data). If GAD:NIMIN1 is part of a complex together with NPR1 and *as-1*-bound TGA factor, the reporter gene is activated and the yeast cells can grow on medium lacking His. As shown in Figure 2A, only in the presence of all three subunits of the ternary complex between TGA2 and TGA6, respectively, NPR1, and GAD:NIMIN1 was reporter gene activation observed and were yeast cells able to grow on medium lacking His (sections 5 and 7). These data demonstrate the interaction between NPR1 and DNA-bound TGA factor together with GAD:NIMIN1 in an *in vivo* system (Figure 2B). The data indicate that an assembly of the ternary complex on the *PR-1* promoter is possible.

#### Constitutive Expression of NIMIN1 but Not of NIMIN1-2 Leads to Repression of *PR* Gene Expression after SA Induction

SA and benzo(1,2,3)thiadiazole-7-carbothioic acid inducibility of *NIMIN1*, and the interaction between NIMIN1, NPR1, and TGA factors in yeast, prompted us to test whether the expression of SAR marker genes is altered in *35S:NIMIN1* lines. We examined the expression of *PR* genes in a time-course experiment 0, 4, 8, and 24 h after SA treatment. As shown by the RNA gel blot in Figure 3A, SA-induced expression of *PR-1*, *PR-2*, and *PR-5* was strongly reduced in lines with high levels of NIMIN1:myc:His<sub>6</sub> expression (similar results were obtained with all lines showing NIMIN1:myc:His<sub>6</sub> expression presented in Figure 1A). Interestingly, induction of the endogenous *NIMIN1* transcript was also repressed. This reduction was not observed in lines with low levels of NIMIN1:myc:His<sub>6</sub>. To corroborate our findings, we tested whether *PR* genes are also repressed in *35S:NIMIN1* lines using an independent inducing stimulus. *35S:NIMIN1* lines were challenged with the avirulent bacterial strain *Pseudomonas syringae* pv *maculicola* ES4326 *avrRpt2* (*Psm* 4326 *avrRpt2*) (Dong et al., 1991; Whalen et al., 1991), and *PR* gene expression was examined 0, 8, 12, and 24 h after infection. As shown in Figure 3B, *Psm* 4326 *avrRpt2*-induced expression of SAR genes was reduced in lines with high levels of NIMIN1:myc:His<sub>6</sub>, whereas *PR-2* and *PR-5* were less strongly repressed than *PR-1*. Again, induction of the endogenous *NIMIN1* transcript was also repressed. In lines with low levels of NIMIN1:myc:His<sub>6</sub>, *PR* genes were induced comparably with the wild type. These results indicate that NIMIN1 may be a negative regulator of *PR* gene expression.

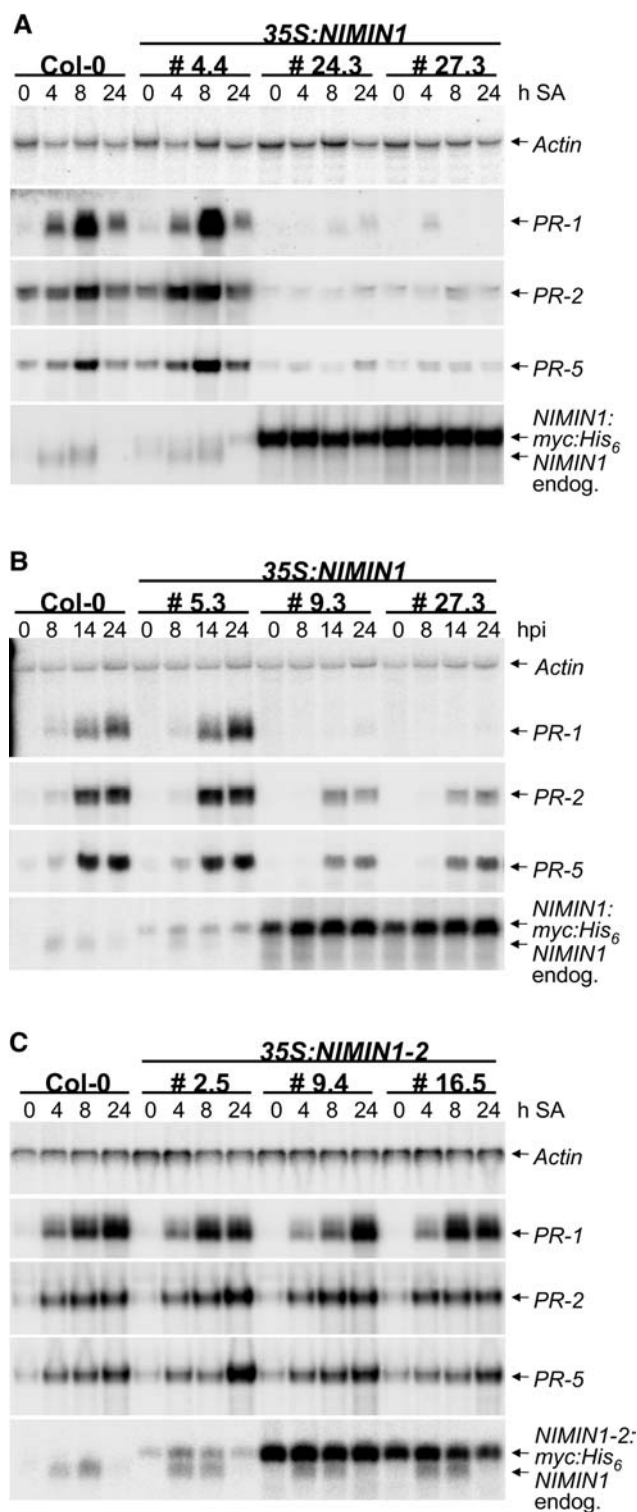
To test whether *PR* gene repression is contingent on the ability of NIMIN1 to interact with NPR1, we investigated *PR* gene expression in *35S:NIMIN1-2* lines in a time-course experiment 0, 4, 8, and 24 h after SA treatment. As shown by RNA gel blot analysis in Figure 3C, *PR* gene expression in *35S:NIMIN1-2* plants did not differ from that in wild-type plants, although NIMIN1-2 was as highly expressed as NIMIN1 (Figure 1D). Induction of the endogenous *NIMIN1* transcript also was not



**Figure 2.** A Ternary Complex of NIMIN1, NPR1, and TGA Factor Binds the *as-1* Promoter Element in the Yeast One-Hybrid System.

**(A)** Cells of the yeast strain yTSH1 carrying a  $3 \times as-1:HIS3$  reporter gene construct were cotransformed with the respective plasmids encoding interaction partners. Transformants were grown on selective medium lacking Leu, Trp, and uracil (data not shown). Expression of NPR1 and TGA factors was under the control of the *Met-25* promoter. GAD:NIMIN1 was expressed under the control of the *CUP1* promoter. To test for protein-DNA interaction, similar amounts of cells were transferred on medium lacking Leu, Trp, uracil, and His in the absence of Met. The proteins expressed in yeast cells that were spotted on the different sectors of the plate are listed in the table. Cells expressing only one or two subunits of the ternary DNA binding complex were transferred to sectors 1 to 4, 6, and 8. They served as negative controls. Only in the presence of all three subunits of the ternary complex between TGA2 and TGA6, NPR1, and GAD:NIMIN1 was reporter gene activation observed and were yeast cells able to grow on medium lacking His (sections 5 and 7).

**(B)** Scheme of the ternary complex formed by GAD:NIMIN1, NPR1, and TGA factors bound to an *as-1* element of the *HIS3* reporter gene construct in yTSH1 cells.



**Figure 3.** PR Gene Expression in Plants Overexpressing NIMIN1 and NIMIN1-2.

**(A)** RNA gel blot analysis of PR gene expression in wild-type and 35S:NIMIN1 lines in response to treatment with SA. 35S:NIMIN1 line 4.4 expresses NIMIN1:myc:His<sub>6</sub> to low levels, whereas lines 24.3 and 27.3

are affected. This result shows that PR gene and NIMIN1 repression in 35S:NIMIN1 lines depends on the ability of NIMIN1 to interact with NPR1. Constitutive expression of NIMIN1:myc:His<sub>6</sub> in the wild-type background may antagonize NPR1 activity and lead to a phenotype like that conferred by *npr1*.

#### SAR Is Abolished in 35S:NIMIN1 Lines

To investigate whether 35S:NIMIN1 lines have additional *npr1*-like characteristics, we tested whether the loss of SA-induced PR gene expression would coincide with impaired SAR-mediated protection against infection with a virulent pathogen. Two-week-old plants (35S:NIMIN1 lines 9.3 and 27.3, *npr1-1*, and Col-0) were inoculated with *P. syringae* pv *maculicola* ES4326 (*Psm* 4326; OD<sub>600</sub> = 0.2) (Dong et al., 1991; Whalen et al., 1991) with or without pretreatment with 1 mM SA or were mock inoculated (Figure 4A). Without pretreatment with SA, all plant lines tested were infected by the virulent bacterial strain and showed severe disease symptoms. Pretreatment with SA protected wild-type plants as a result of the establishment of SAR. In *npr1-1* plants, however, SAR was abolished. They showed strong disease symptoms when infected with a virulent bacterial strain even after SAR-inducing treatment. Similarly, 35S:NIMIN1 lines 9.4 and 27.3 showed strong disease symptoms after SA pretreatment, indicating that the repression of SA-inducible PR gene expression coincides with an impaired SAR response. This is a second common feature of 35S:NIMIN1 lines and *npr1* mutants. To confirm that the macroscopic disease symptoms reflect the bacterial growth inside the plant, we measured the bacterial titer in SA-pretreated wild-type, 35S:NIMIN1 line 27.3, and *npr1-1* plants at 0 and 3 d after infection (DAI). In wild-type plants, the *Psm* 4326 titer increased only ~10-fold after 3 DAI. By contrast, 35S:NIMIN1 line 27.3 showed an increase of approximately three orders of magnitude, similar to the situation in *npr1-1* plants. This is in accordance with the observed macroscopic disease symptoms and with published data (Cao et al., 1994).

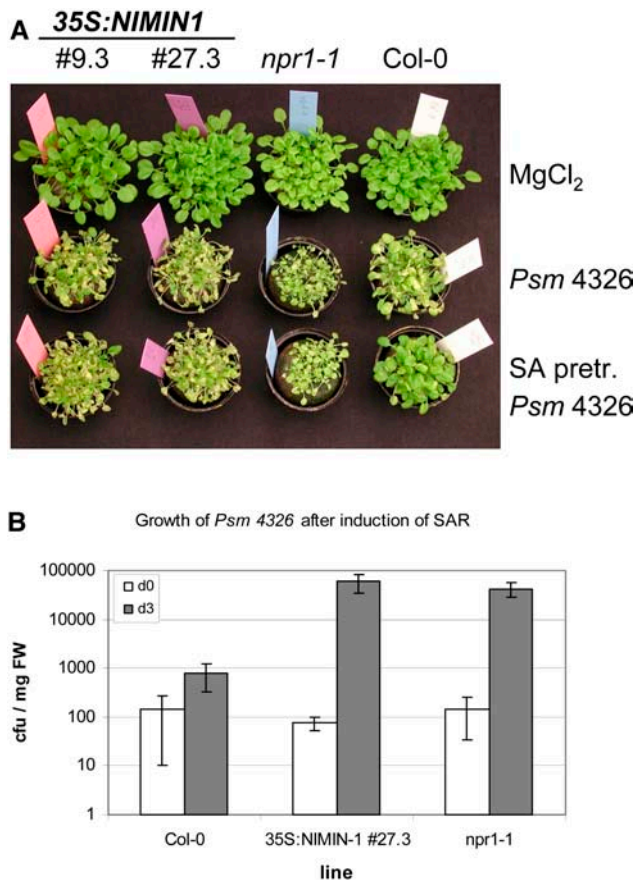
#### 35S:NIMIN1 but Not 35S:NIMIN1-2 Lines Are Susceptible to an Avirulent Bacterial Strain

To examine whether 35S:NIMIN1 lines are impaired not only in SAR but also in R gene-mediated resistance, we infected

express NIMIN1:myc:His<sub>6</sub> to high levels. Total RNA was extracted from 3-week-old plants kept under short-day conditions (21/19°C, 8-h-light/16-h-dark cycle, and 60% relative humidity) at 0, 4, 8, and 24 h after SA treatment. Detection of ACTIN mRNA served as a loading control.

**(B)** Expression of PR genes in wild-type and 35S:NIMIN1 lines in response to bacterial infection. 35S:NIMIN1 line 5.3 expresses NIMIN1:myc:His<sub>6</sub> to low levels, whereas lines 9.3 and 27.3 express NIMIN1:myc:His<sub>6</sub> to high levels. Total RNA was extracted from 3-week-old plants at 0, 8, 14, and 24 h after *Psm* 4326 *avrRpt2* infection. Detection of ACTIN mRNA served as a loading control.

**(C)** Expression of PR genes in 35S:NIMIN1-2 lines in response to treatment with SA. 35S:NIMIN1-2 line 2.5 expresses NIMIN1-2:myc:His<sub>6</sub> to low levels, whereas lines 9.4 and 16.5 express NIMIN1-2:myc:His<sub>6</sub> to high levels. Total RNA was extracted from 3-week-old plants at 0, 4, 8, and 24 h after SA treatment. Detection of ACTIN mRNA served as a loading control.



**Figure 4.** Response of 35S:NIMIN1, *npr1-1*, and Wild-Type Lines to *Psm 4326* Infection after SA Pretreatment.

**(A)** Macroscopic symptoms of 35S:NIMIN1 lines 9.3 and 27.3, *npr1-1*, and Col-0 plants 4 d after inoculation with *Psm 4326*. Plants were mock inoculated (top) or infected with *Psm 4326* at an  $OD_{600}$  of 0.2 with (bottom) or without (middle) pretreatment with SA.

**(B)** Growth of *Psm 4326* in wild-type, 35S:NIMIN1 line 27.3, and *npr1-1* plants after pretreatment with SA. Plants were treated with SA before *Psm 4326* infection at an  $OD_{600}$  of 0.002. Samples were taken at 0 and 3 DAI. Three to four samples were taken for each genotype and time point. Data points represent means of three independent experiments  $\pm$  SD. At 3 DAI, significant differences were observed between in planta *Psm 4326* growth in Col-0 plants and 35S:NIMIN1 plants (Student's *t* test;  $P < 0.002$ ) and *npr1-1* plants (Student's *t* test;  $P < 0.003$ ). cfu, colony-forming units.

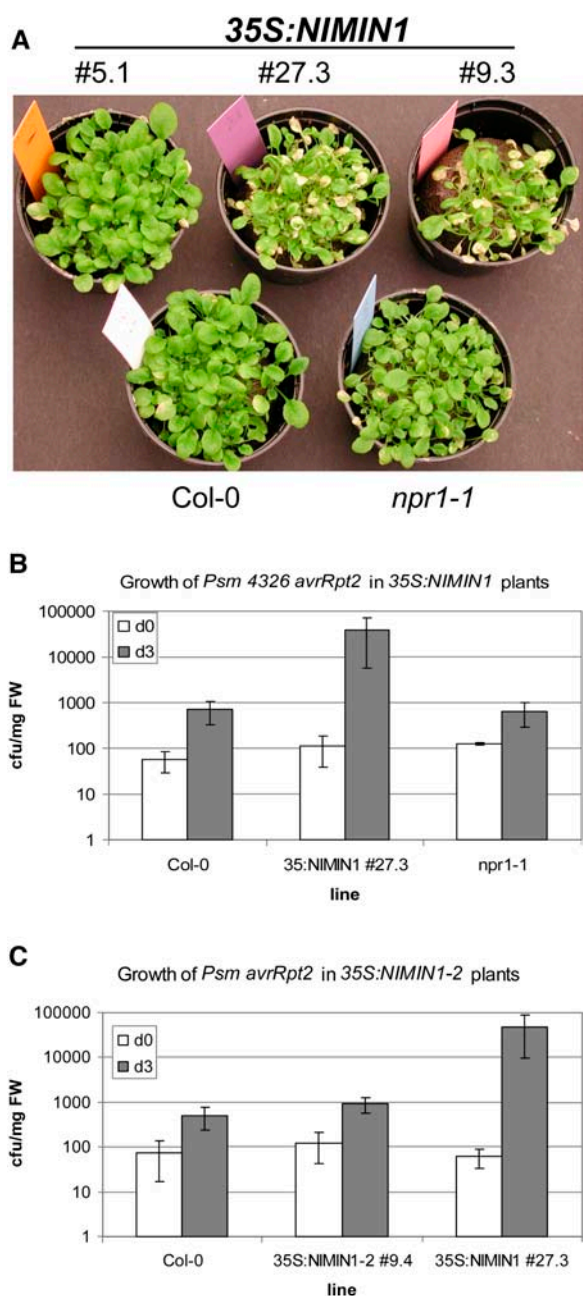
35S:NIMIN1 lines 5.1, 27.3, and 9.3 with the *Psm 4326 avrRpt2* (Dong et al., 1991; Whalen et al., 1991). To observe macroscopic symptoms, plants were dipped in a bacterial suspension ( $OD_{600} = 0.2$ ) and the progress of infection was followed over 5 d. Wild-type and *npr1-1* plants hardly showed any macroscopic signs of infection, as depicted in Figure 5A. 35S:NIMIN1 line 5.1 expressing low levels of NIMIN1:myc:His<sub>6</sub> behaved like a wild-type plant. The Resistance to *Pseudomonas syringae*2 gene confers resistance against *Psm avrRpt2* in these lines. By contrast, 35S:NIMIN1 lines 27.3 and 9.3, with high NIMIN1:myc:His<sub>6</sub> expression, showed a significant number of necrotic leaves compared with wild-type plants, indicating a higher susceptibility even to an

avirulent bacterial pathogen. To confirm that the macroscopic disease symptoms reflect the bacterial growth inside the plant, we measured the bacterial growth in wild-type, 35S:NIMIN1 line 27.3, and *npr1-1* plants at 0 and 3 DAI. As shown in Figure 5B, wild-type and *npr1-1* plants did not support the growth of *Psm 4326 avrRpt2*, as described in the literature (Cao et al., 1994; Clarke et al., 2000). The bacterial titer increased only  $\sim 10$ -fold. By contrast, 35S:NIMIN1 27.3 plants were less well protected. At 3 DAI, these plants showed an  $\sim 85$ -fold higher bacterial titer compared with wild-type plants. These results are in accordance with the observed macroscopic disease symptoms and indicate that overexpression of NIMIN1:myc:His<sub>6</sub> also affects *R* gene-mediated resistance and does not merely lead to a *npr1* phenocopy.

To test whether enhanced susceptibility in 35S:NIMIN1 lines is contingent on the ability of NIMIN1 to interact with NPR1, we investigated the susceptibility of 35S:NIMIN1-2 line 9.4 to *Psm 4326 avrRpt2* compared with wild-type plants and 35S:NIMIN1 line 27.3. Bacterial growth was measured in a titration experiment at 0 and 3 DAI. The data in Figure 5C show that 35S:NIMIN1-2 line 9.4 was as well protected against the avirulent *Psm 4326* strain as the wild-type plants. In both lines, bacterial titers increased  $\sim 10$ -fold at 3 DAI. These results strongly suggest that *PR* gene repression and enhanced susceptibility in 35S:NIMIN1 lines are attributable to the specific interaction between endogenous NPR1 and transgenic NIMIN1 and not to interference of NIMIN1 with a yet unknown factor.

#### A NIMIN1 Knockout Line and NIMIN1 dsRNAi Lines Show Enhanced *PR-1* Gene Induction after SA Treatment

Using a complementary approach to verify that NIMIN1 is a negative regulator of NPR1 activity, a T-DNA insertion line for NIMIN1, designated *nimin1-1* (SALK\_086460), was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). The exact position of the T-DNA insertion was determined by PCR and subsequent sequencing of the T-DNA flanking regions. The T-DNA insertion is 4 bp upstream of the start ATG, as depicted schematically in Figure 6A. Based on PCR and segregation analyses, we obtained progeny homozygous for the T-DNA insertion (*nimin1-1* lines 2 and 7). RNA gel blot analysis revealed that the *nimin1-1* mutant is a real knockout, because no NIMIN1 mRNA was detected after SA treatment, even after extensive exposure of the RNA gel blot, whereas in wild-type plants the transcript had already accumulated strongly at 3 h after SA treatment (Figure 6B). To examine *PR-1* mRNA accumulation in *nimin1-1* knockout lines, the same blot was incubated with a *PR-1* probe. As shown in Figure 6B, *PR-1* mRNA accumulation was stronger in *nimin1-1* lines than in wild-type plants. Quantification of the signals shown in Figure 6B revealed that the difference in *PR-1* signal strength was strongest at 12 h after SA induction (Figure 6C). Compared with Col-0 plants, *nimin1-1* line 2 showed a 2.5-fold increase and *nimin1-1* line 7 showed a 3.3-fold increase in *PR-1* mRNA levels at 12 h after SA induction. Stronger *PR-1* induction in *nimin1-1* lines was reproducible in several experiments with RNAs from independently grown and induced plants. In a time-course experiment with plants harvested at later time points, differences were still present at 24 h after SA induction but were no longer observed



**Figure 5.** Response of 35S:NIMIN1, 35S:NIMIN1-2, *npr1-1*, and Wild-Type Plants to *Psm 4326 avrRpt2* Infection.

**(A)** Macroscopic symptoms of 35S:NIMIN1 lines 5.1, 9.3, and 27.3, Col-0, and *npr1-1* plants 5 d after inoculation with *Psm 4326 avrRpt2*. Plants were infected with *Psm 4326 avrRpt2* at an  $OD_{600}$  of 0.2. In 35S:NIMIN1 line 5.1, NIMIN1:myc:His<sub>6</sub> is not detectable (Figure 1A).

**(B)** Growth of *Psm 4326 avrRpt2* in wild-type, 35S:NIMIN1 line 27.3, and *npr1-1* plants. Plants were infected with *Psm 4326 avrRpt2* at an  $OD_{600}$  of 0.002. Samples were taken at 0 and 3 DAI. Three to four samples were taken for each genotype and time point. Data points represent means of three independent experiments  $\pm$  SD. At 3 DAI, significant differences were observed between in planta *Psm 4326 avrRpt2* growth in Col-0 (Student's *t* test;  $P < 0.005$ ) and *npr1-1* plants (Student's *t* test;  $P < 0.005$ ) compared with 35S:NIMIN1 plants. cfu, colony-forming units.

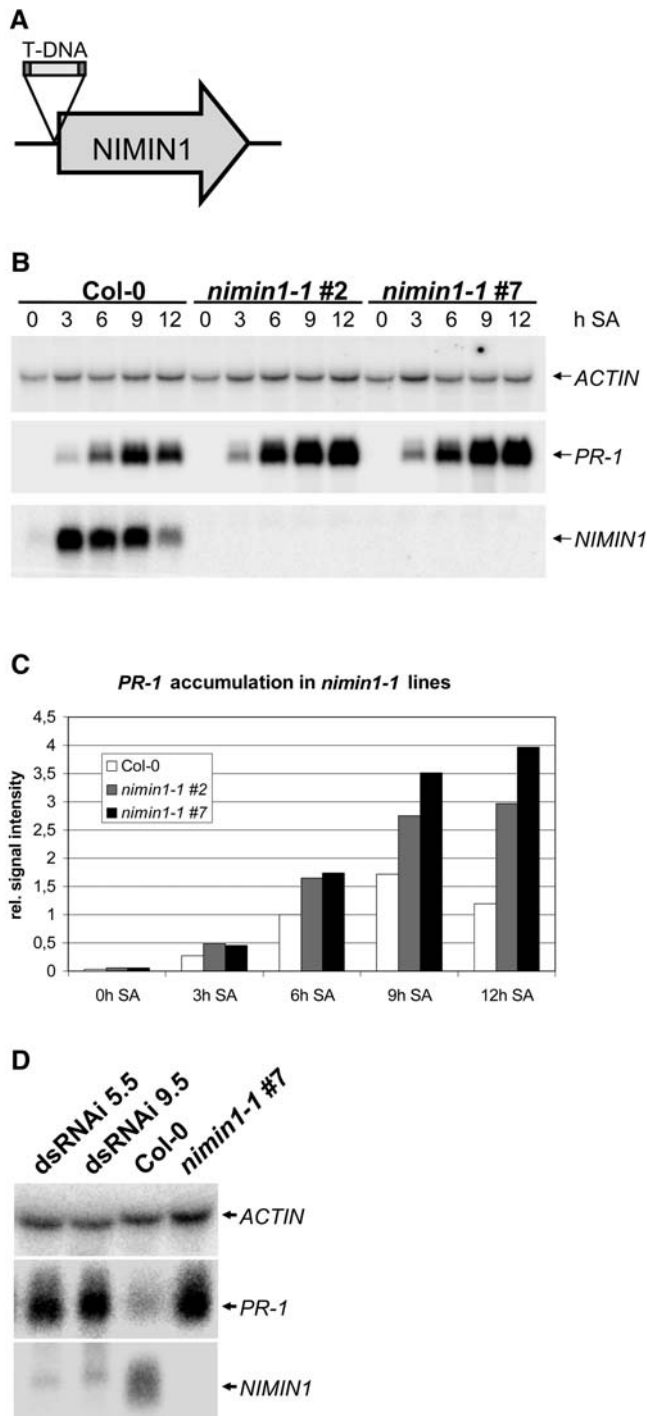
after 36 or 48 h (data not shown), indicating that the amplitude but not the shutting down of *PR-1* gene expression is affected in *nimin1-1*. Basal *PR-1* expression was not notably different in *nimin1-1* lines compared with wild-type plants.

To verify that the mutant phenotype is specifically attributed to the T-DNA insertion in *NIMIN1* and not to unrelated T-DNA insertions or point mutations that are known to be generated as part of the transformation process (McElver et al., 2001), we generated dsRNAi transgenic lines to silence the *NIMIN1* gene. A second *NIMIN1* T-DNA insertion line was not available from the Nottingham Arabidopsis Stock Centre. First, we tested *NIMIN1* mRNA levels in *NIMIN1* dsRNAi lines compared with wild-type and *nimin1-1* lines at 8 h after SA induction (all plant lines were grown and treated in parallel). RNA gel blot analysis revealed that *NIMIN1* expression was strongly reduced in 63 of 74 individuals of nine independent *NIMIN1* dsRNAi lines. Figure 6D shows representative results for two individuals of two independent T2 lines. Next, *PR-1* gene expression was tested using the same blots. Compared with the wild type, *PR-1* mRNA levels were higher in *NIMIN1* dsRNAi lines and similar to the level of a *nimin1-1* line (Figure 6D). These results strongly support the hypothesis that NIMIN1 counteracts NPR1 activity and modulates *PR* gene expression. To test whether stronger *PR-1* gene expression in *nimin1-1* plants coincides with enhanced resistance against a bacterial pathogen, the mutant was subjected to infection with both *Psm 4326 avrRpt2* and *Psm 4326* with and without SAR-inducing treatment. Using our experimental setup, no significant changes in the defense responses of *nimin1-1* plants were observed (data not shown), indicating that hyperactivation of *PR-1* gene expression does not coincide with enhanced resistance.

## DISCUSSION

In addition to members of the TGA family of basic domain/leucine zipper transcription factors, NIMIN proteins have been found to interact with NPR1 in several independent two-hybrid screens (Weigel et al., 2001). Compared with the TGA factors, their mechanism of action is much less obvious. Before we started to analyze the biological function of the NIMIN proteins, we verified the interaction between NPR1 and NIMIN1 in plant extracts. NIMIN1 was chosen because it exhibits the strongest affinity with NPR1 compared with other NIMIN proteins (Weigel et al., 2001). We created transgenic plants expressing *NIMIN1* under the control of the 35S promoter in the wild-type background (35S:NIMIN1 plants). As a control, transgenic plants were created expressing a mutant designated *NIMIN1-2* (35S:NIMIN1-2

**(C)** Bacterial growth in 35S:NIMIN1-2 plants compared with wild-type and 35S:NIMIN1 plants. Col-0, 35S:NIMIN1-2 line 9.4, and 35S:NIMIN1 line 27.3 plants were infected with *Psm 4326 avrRpt2* at an  $OD_{600}$  of 0.002. Samples were taken at 0 and 3 DAI. Three to four samples were taken for each genotype and time point. Data points represent means of three independent experiments  $\pm$  SD. At 3 DAI, significant differences were observed between in planta *Psm 4326 avrRpt2* growth in Col-0 compared with 35S:NIMIN1 plants (Student's *t* test;  $P < 0.03$ ) and 35S:NIMIN1-2 plants (Student's *t* test;  $P < 0.03$ ). cfu, colony-forming units.



**Figure 6.** Characterization of a *NIMIN1* Knockout Line *nimin1-1* and *NIMIN1* dsRNAi Lines with Respect to *PR-1* Gene Expression.

**(A)** Scheme of the *nimin1-1* locus. *nimin1-1* carries a T-DNA insertion 4 bp upstream of the start codon of *NIMIN1*.

**(B)** RNA gel blot analysis of *PR-1* and *NIMIN1* gene expression in wild-type and *nimin1-1* lines 2 and 7. Total RNA was extracted from 3-week-old plants at 0, 3, 6, 9, and 12 h after SA treatment. Detection of *ACTIN* mRNA served as a loading control.

plants) that is unable to interact with NPR1 in the yeast two-hybrid system. *NIMIN1-2* carries two amino acid exchanges (F49S and F50S) in the NPR1 interaction domain (Weigel et al., 2001). To facilitate the purification and detection of the proteins, a c-myc:His<sub>6</sub> tag was fused to the C termini, resulting in the expression of *NIMIN1:myc:His<sub>6</sub>* and *NIMIN1-2:myc:His<sub>6</sub>* fusion proteins. Homozygous plants expressing comparable amounts of the transgene were crossed with homozygous plants expressing a HA-tagged NPR1 under the control of the 35S promoter (35S:

*NPR1*) in the *npr1-1* background. Using nickel affinity chromatography, we were able to copurify NPR1 from SA-treated F1 plants expressing HA<sub>3</sub>:NPR1 and *NIMIN1:myc:His<sub>6</sub>* but not from F1 plants expressing HA<sub>3</sub>:NPR1 and *NIMIN1-2:myc:His<sub>6</sub>*, verifying our yeast data and demonstrating *NIMIN1*–NPR1 interaction in plant extracts.

### The Role of *NIMIN1* in Gene Expression

Transient expression of *NIMIN* mRNAs after SA treatment and the formation of a ternary complex between *NIMIN* proteins, NPR1, and TGA factors suggested a function of the *NIMIN* proteins in SA-mediated *PR* gene regulation. Therefore, we tested whether altered *NIMIN1* expression would affect *PR* gene expression. *NIMIN1* knockout mutant *nimin1-1* and *NIMIN1* dsRNAi lines showed enhanced *PR-1* accumulation after SA treatment, clearly demonstrating a negative effect of *NIMIN1* on *PR-1* expression. Accordingly, *PR* gene activation was strongly suppressed in 35S:*NIMIN1* plants after SA treatment or infection with an avirulent bacterial strain. This effect was not observed in plants constitutively expressing *NIMIN1-2*, thus demonstrating that *NIMIN1* exerts its negative effect through its interaction with NPR1. Consistently, 35S:*NIMIN1* plants mimic the known *npr1* phenotype. On the other hand, *nimin1-1* lines show a phenotype similar to that of 35S:*NPR1* plants, at least with regard to *PR-1* expression (Cao et al., 1998): both plant lines are characterized by an approximately threefold higher *PR-1* expression after SA induction. Thus, higher amounts of active NPR1 can be made available, either by enhancing NPR1 protein levels or by reducing the amount of the negative regulator *NIMIN1*. This positive effect on gene expression can only be exerted in the presence of SA, as NPR1 is located in the cytosol in the uninduced state (Kinkema et al., 2000). This inactive condition is independent of the relative amounts of NPR1 and *NIMIN1*, as shown by unaltered *PR-1* expression in the uninduced state in *nimin1-1* and 35S:*NPR1* plants. The difference in *PR-1* expression between wild-type and *nimin1-1* lines was observed until 24 h after induction. At later time points, the difference was negligible, probably because of the lack of *NIMIN1* in wild-type plants during this late state, when

**(C)** Quantification of *PR-1* mRNA accumulation based on the data shown in **(B)**. The graph depicts levels of *PR-1* mRNA normalized to that of *ACTIN*.

**(D)** RNA gel blot analysis of *PR-1* and *NIMIN1* gene expression in individuals of dsRNAi lines 5.5 and 9.5, a wild-type plant, and *nimin1-1* line 7. Total RNA was extracted from 2-week-old plants at 8 h after SA treatment. Detection of *ACTIN* mRNA served as a loading control.



		EAR motif	
NIMIN1	(130-142)	RNEDKALDLN <b>LAL</b>	-----
NIMIN1b	(128-140)	MRKDNGLDLN <b>LAL</b>	-----
NIMIN2	(101-122)	GLQGLGLDLN <b>CKPEP</b> DSVLSL	-----
NIMIN3	(100-112)	SETNVCLDLN <b>L</b> SL	-----
G8-1	(67-85)	KRVVPGKDLN <b>LNT</b> LPGNGD	-----
AtERF3	(193-225)	RNPPFQFDLN <b>FPP</b> LDCVDLFGNADDLHCTDLRL	-----
AtERF4	(205-222)	RSQLLDLDLN <b>L</b> PPPSEQA	-----
NtERF3	(209-225)	GKRGIDLDLN <b>L</b> APPMEF	-----
ZAT1	(243-267)	GIIISLIDLN <b>L</b> PAPSEEEEMASSVF	-----
ZAT10	(183-227)	SSSHRGFDLN <b>I</b> PP <b>I</b> PEFSMVNGDDEVMSMPAKKPRFDFPVKLQL	-----
ZAT11	(139-174)	SKRI <b>L</b> SLDLN <b>L</b> TPLENDLEYIFGKTFVVKIDMKFVL	-----
Consensus		L LDLNL P E	

**Figure 7.** The EAR Motif Is Conserved in the C-Terminal Regions of NIMIN, Class II ERF, and TFIIIA-Type Zinc Finger Proteins.

Comparison of the amino acid sequences of the C-terminal regions of NIMIN, class II ERF, and TFIIIA-type zinc finger proteins from Arabidopsis and tobacco. Numbers in parentheses indicate the positions of amino acid sequences. The EAR motif [ $^{1-7}$ DLN $^{1-7}$ (x)P] and other conserved amino acids are shaded. G8-1, NIMIN2 homolog of tobacco encoded by a previously described cDNA (Horvath et al., 1998).

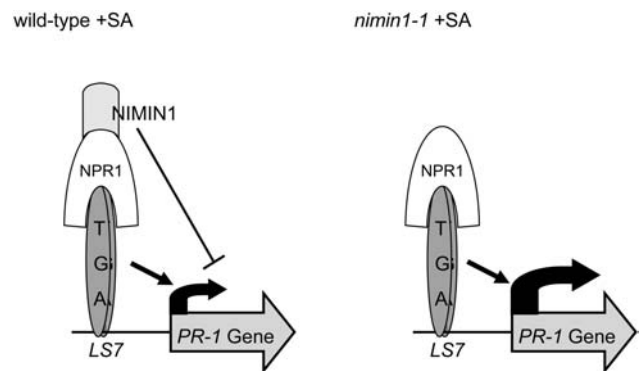
*NIMIN1* transcription is shut down. Thus, *NIMIN1* is involved in neither turning on nor turning off *PR-1* expression. Rather, it modulates its amplitude. This function seems to be specific for *NIMIN1*, because it cannot be replaced by the other three members of the NIMIN family, albeit it is possible that in the absence of *NIMIN1* other NIMIN proteins may partially take over its function. Interestingly, transcript levels of the endogenous *NIMIN1* gene were reduced in *35S:NIMIN1* but not in *35S:NIMIN1-2* plants, suggesting that *NIMIN1* regulates its own expression through its interaction with NPR1. Differential expression analysis using microarrays and RNA of induced wild-type and knockout plants will provide a suitable tool to identify additional genes regulated by *NIMIN1*.

The mechanism by which *NIMIN1* represses *PR* gene expression after SA induction remains to be analyzed. The fact that *NIMIN1* was found to bind simultaneously with NPR1 and TGA factors to a *PR-1* promoter element in an *in vivo* situation suggests that it acts as a transcriptional repressor. Two types of transcriptional repressors have been described: passive repressors, which directly bind to DNA, inhibiting access of transcriptional activators; and active repressors, which repress transcription through a distinct repression domain (Hanna-Rose and Hansen, 1996). The primary structure of *NIMIN1* shows no homology with known DNA binding domains, suggesting that *NIMIN1* may act as an active repressor after being recruited to the DNA/TGA/NPR1 complex. Interestingly, the previously described C-terminal LXL motif of *NIMIN1* reveals striking similarity to the so-called ethylene-responsive element binding factor (ERF)-associated amphipathic repression motif [EAR;  $^{1-7}$ DLN $^{1-7}$ (x)P] identified in ERF and zinc finger transcription factors (Figure 7) (Ohta et al., 2001). Recently, an EAR motif was also identified in Auxin/Indole-3-Acetic Acid repressors (Tiwari et al., 2004). These repressors are unable to bind to DNA themselves but are recruited to the DNA through their interaction with ARF transcriptional activators, which are rendered inactive through the action of the EAR motif. Similarly, *NIMIN1* might bind to the NPR1/TGA factor complex, repressing or reducing *PR* gene expression (Figure 8). The assembly of a ternary complex of *NIMIN1*, NPR1, and TGA factors on an SA-responsive promoter

element in the yeast one-hybrid system supports this view. All four Arabidopsis *NIMIN* proteins contain this putative EAR domain (Figure 7). Their low overall sequence similarity might enable them to interact with different additional proteins, facilitating their recruitment to different promoters. Through this mechanism, distinct subsets of NPR1-dependent genes might be regulated by different *NIMIN* proteins.

### The Role of *NIMIN1* in Disease Susceptibility

Transgenic plants encoding *35S:NIMIN1* were severely compromised with respect to mounting SAR after SA treatment. Thus, *35S:NIMIN1* plants mimicked the phenotype conferred by *npr1*,



**Figure 8.** Proposed Model for the Regulatory Mechanism of SA-Mediated *PR-1* Gene Expression.

In wild-type plants, NPR1 forms a ternary complex with *NIMIN1* and TGA factors upon SAR induction that binds to a positive regulatory *cis* element of the *PR-1* promoter, termed *LS7* (Lebel et al., 1998). This leads to *PR-1* gene induction. *NIMIN1* decreases transcriptional activation, possibly through its EAR motif, which results in fine-tuning of *PR-1* gene expression. In the *nimin1-1* mutant, *NIMIN1* is absent from the transactivating protein complex upon SAR induction, which leads to enhanced *PR-1* gene expression caused by the lack of the repressing function of *NIMIN1*.

as already observed for *PR-1* gene expression. However, enhanced inducible *PR-1* induction in *nimin1-1* lines did not coincide with enhanced resistance. In this regard, these plants differ from the *35S:NPR1* plants, which are more resistant to the virulent bacterial strain *Psm* 4326 (Cao et al., 1998). This finding is consistent with the above-mentioned hypothesis that NIMIN1 negatively regulates only a subset of NPR1-dependent genes. The enhanced expression of this subset in *nimin1-1* plants may not be sufficient for enhanced resistance against *Psm* 4326. Alternatively, the closest relative of NIMIN1, designated NIMIN1b (Weigel et al., 2001), which shares 44% identity and 64% similarity, may partially take over its function in *nimin1-1* plants.

The impaired defense observed in *35S:NIMIN1* plants is presumably attributable to high amounts of the NPR1/NIMIN1 complex, leading to severely reduced amounts of active NPR1. It can further be speculated that high levels of NIMIN1 compete with other members of the NIMIN family. Thus, additional subsets of NPR1-dependent genes may be downregulated in *35S:NIMIN1* plants, finally leading to a compromised SAR. T-DNA insertion lines are available for *NIMIN1b*, *NIMIN2*, and *NIMIN3*. They are currently used to generate double and triple knockouts to test whether the lack of multiple members of the NIMIN family can lead to enhanced disease resistance.

In contrast with their *npr1*-like features, *35S:NIMIN1* plants are impaired in *R* gene-mediated resistance against *Psm* 4326 *avrRpt2*. This is not found in the *npr1-1* mutant (Cao et al., 1994; Clarke et al., 2000), an observation that has led to the conclusion that NPR1 plays only a minor role in local defense responses against avirulent pathogens (Clarke et al., 2000). This apparent contradiction can be resolved by assuming that *35S:NIMIN1* plants mimic an *npr1* allele that is even stronger than the ones described to date. Alternatively, NIMIN1 but not NIMIN1-2 may interact with NPR1-like proteins present in *Arabidopsis* that possibly play a role in *R* gene-mediated resistance. It also can be speculated that for some functions the lack of *npr1* does not have the same consequences as the presence of an NPR1/NIMIN1 complex, which might actively inhibit *R* gene-mediated resistance, providing a negative feedback control device. Either way, our data clearly indicate that NPR1 or NPR1-like proteins play a role in *R* gene-mediated resistance in *Arabidopsis*. Therefore, the dominant negative effect of constitutively expressed NIMIN1 on NPR1 has revealed a yet unknown function of NPR1. Similar observations were made in Solanaceae: NPR1-like proteins were shown to be essential for *N* gene-mediated resistance against *Tobacco mosaic virus* in tobacco (Liu et al., 2002) and for *Pto*-mediated resistance against *Pseudomonas syringae* pv *tomato* DC3000 *avrPto* in tomato (Ekengren et al., 2003).

In conclusion, we have shown that NIMIN1 can counteract NPR1-activated gene expression. However, only a subset of NPR1-dependent genes may be hyperactivated in the absence of NIMIN1, which is not sufficient to increase plant resistance. As mentioned above, simultaneous knockout of several NIMIN genes might lead to the same phenotype as revealed by *35S:NPR1* plants. Negative regulators of NPR1, such as NIMIN1, might ensure that plant resources are not allocated to the defense response to an extent that would adversely affect other fitness-relevant processes, such as growth and reproduction (Heil and

Baldwin, 2002). Single PR proteins may account for ~1% of the total soluble protein in infected tobacco plants (Antoniw and Pierpoint, 1978), indicating that large quantities of resources can be allocated to resistance traits. SA- and JA-dependent signal transduction pathways mutually inhibit each other (Kunkel and Brooks, 2002), also suggesting the necessity for shutting down certain defense responses to avoid exhaustion of resources. As a matter of fact, NPR1 is involved in the suppression of JA-dependent genes in the presence of SA (Spoel et al., 2003). In the absence of NIMIN proteins, hyperactivated NPR1 may lead to enhanced repression of JA-mediated protection, rendering plants even more susceptible to many necrotrophic pathogens (Heil and Baldwin, 2002). Thus, NIMIN proteins might be indirectly involved in the distribution of resources to either SA/JA-mediated defense responses or processes relevant for growth and reproduction. Given that plants are exposed to a complex environment, different NIMIN proteins might adjust the amplitude of activation of subsets of defense responses depending on the prevailing biotic and abiotic conditions.

## METHODS

### DNA Constructs

To generate constructs for expression in *Arabidopsis thaliana*, *NIMIN1* was cloned as a *Bam*HI fragment into the *Bgl*III-cleaved vector pTrcHis2c (Invitrogen, Karlsruhe, Germany), creating an in frame fusion with a c-myc and the His<sub>6</sub> epitope tag. The *NIMIN1:myc:His<sub>6</sub>* sequence was cut out as a *Bam*HI/*Pme*I fragment and ligated to *Bam*HI/*Sma*I-cut pUC19. *NIMIN1:myc:His<sub>6</sub>* was then cut out as a *Bam*HI/*Sac*I fragment and cloned into the *Bam*HI/*Sac*I-cut vector pUC19/35S:β-glucuronidase (Beilmann et al., 1991), resulting in the plasmid pUC19/35S:NIMIN1:myc:His<sub>6</sub>. From there, the 35S:NIMIN1:myc:His<sub>6</sub> cassette was cloned as an *Eco*RI fragment into the *Eco*RI-opened vector pBIN19/35S:β-glucuronidase, resulting in the plasmid pBIN19/35S:NIMIN1:myc:His<sub>6</sub>.

To create the *NIMIN1-2* allele, the N terminus of *NIMIN1* was amplified by PCR using oligonucleotides pUC19bck (5'-TGTGGAATTGTGAGC-GGA-3') and N1-M1 (5'-GGAAGCTTAGAGGACGTATCAATCTTC-3') and pUC19/NIMIN1:myc:His<sub>6</sub> as a template. Amplification with N1-M1 results in a DNA fragment coding for two amino acid exchanges (F49S and F50S) in the NPR1 interaction domain of NIMIN1 (Weigel et al., 2001). The amplified PCR fragment was cut with *Hind*III and then cloned into the *Hind*III-cut vector pUC19/NIMIN1:myc:His<sub>6</sub>. pBIN19/35S:NIMIN1-2:myc:His<sub>6</sub> was cloned as described for pBIN19/35S:NIMIN1:myc:His<sub>6</sub>. To create a binary vector for the expression of a tagged version of NPR1 under the control of the 35S promoter, Gateway technology (Invitrogen) was used. The full-length cDNA of NPR1 was amplified using primers 5'-GGGGACAAGTTTGTACAAAAAGCAGGCTCCATGGACACCACCA-TTGATGG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCACCGACGACGATGAGAGAG-3'. The generated PCR product was recombined into pDONR207 (Invitrogen). The resulting plasmid pDONR207/NPR1 was further recombined into pAlligator2 (Bensmihen et al., 2004). To generate *NIMIN1* dsRNAi lines, the coding sequence was amplified using primers 5'-GGGGACAAGTTTGTACAAAAAGCAGGCTATATGTATCCTAAACAATTTAG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCACCAAGAAAGCTGGGTCCAATGCAAGATTAAGATC-3'. The amplification product was recombined into the donor vector pDONR207 using the Gateway system (Invitrogen). A second recombination step with a Gateway-compatible derivative of pFGC5491 (Kerschen et al., 2004) yielded the binary construct pFGC5941/NIMIN1, containing a chalcone synthase intron designed to produce double-stranded RNA in plants.

*NIMIN1* was recombined upstream and downstream of the chalcone synthase intron in both the antisense and sense orientations. All DNA constructs were verified by dideoxynucleotide sequencing.

To create the 3× *as-1* yeast reporter gene construct, two complementary oligonucleotides [5'-AATTC(TGACGTAAGGGATGACGCAT-TACC)<sub>3</sub>T-3' and 5'-CTAGA(GGTAATGCGTCATCCCTTACGTCA)<sub>3</sub>G-3'] were aligned, creating *EcoRI*-compatible ends at the 5' end and *XbaI*-compatible ends at the 3' end. The resulting double-stranded DNA fragment was cloned into the plasmid pHisI-1 (BD Biosciences, Heidelberg, Germany). Integration, selection, and analysis of the resulting yeast strain yTSH1 (a derivative of YM4271) were performed as described by the manufacturer (BD Biosciences). To express the TGA factors in yeast, their corresponding cDNAs were cloned as *Bam*HI fragments into the *Bgl*III site of a modified pBridge plasmid (Weigel et al., 2001), resulting in the plasmids pGBD-/TGA2 and pGBD-/TGA6, respectively (TRP1-selectable marker). To express NPR1 in yeast, the Met-25 expression cassette of pGBD-/NPR1 (Weigel et al., 2001) was cloned as a *Stu*I fragment into the *Pvu*II-cleaved pGAD424 plasmid (BD Bioscience), resulting in the plasmid pGAD-/NPR1 (LEU2-selectable marker). The GAD:NIMIN1 fusion was cloned as described in Weigel et al. (2001) for the GAL4BD fusion, resulting in the plasmid pGAD424/NIMIN1. To express the fusion in yeast under the control of the CUP1 promoter, pGAD424/NIMIN1 was partially digested with *Hind*III. The appropriate fragment was cloned into the *Hind*III-cleaved pCU426 plasmid (Labbe and Thiele, 1999), resulting in the plasmid pCU426/GAD:NIMIN1 (URA3-selectable marker).

### Plant Growth Conditions and Transformation

Arabidopsis plants (ecotype Col-0) were grown in soil under controlled environmental conditions (21/19°C, 16-h-light/8-h-dark cycle, and 60% relative humidity unless stated otherwise). All seeds were vernalized at 4°C for 2 d before placement in a growth environment. For pathogen infection, 10 to 20 plants were grown on the mesh of Jiffy-7 peat pots. To maintain high humidity, plant trays were covered with a lid throughout the entire growth period.

Binary plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0 plants using a floral dipping method (Clough and Bent, 1998). Transgenic plants containing pBIN19 constructs were selected on MS agar plates containing 50 µg/mL kanamycin. To generate homozygous transgenic lines, T0 plants were selfed and the progeny of T1 plants were analyzed on MS plates containing 50 µg/mL kanamycin. If 100% of 50 to 100 T2 seedlings were resistant to kanamycin, the T1 mother plant was scored as homozygous. To generate plants expressing HA<sub>3</sub>:NPR1 under the control of the 35S promoter, *npr1-1* plants were transformed with pAlligator/NPR1. Transgenic seeds containing pAlligator/NPR1 were selected using the seed-specific green fluorescent protein (GFP) marker. If all of the seeds of a T1 siliqua were GFP-positive, the plant was scored as homozygous. To create plants expressing HA<sub>3</sub>:NPR1 and NIMIN1:myc:His<sub>6</sub> or NIMIN1-2:myc:His<sub>6</sub>, 35S:NIMIN1 line 27.3 or 35S:NIMIN1-2 line 9.4, respectively, was crossed with a 35S:NPR1 line (pollen donor). GFP-positive F1 seed was used to grow plants for the coaffinity purification experiment. Transgenic plants containing pFGC5941/NIMIN1 constructs were selected by spraying a solution of Basta (glufosinate) herbicide according to the manufacturer's instructions (AgrEvo, Dueseldorf, Germany).

### Protein Gel Blot Analysis

Total denaturing protein extracts were made from Arabidopsis plants by grinding plants in liquid nitrogen and adding two volumes of denaturing urea buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, and 8 M urea, pH 8.0). The extracts were cleared of cell debris by centrifugation at 16,000g at

4°C. Extracts contained equal amounts of proteins, as confirmed by Coomassie Brilliant Blue G 250 staining of a protein gel. His<sub>6</sub>-tagged proteins were enriched by affinity chromatography using Ni-NTA as described by the manufacturer (Qiagen, Hilden, Germany). Extracts were separated and transferred to nitrocellulose membranes according to Sambrook et al. (1989).

Subsequently, the blots were probed with an antibody against the c-myc tag (New England Biolabs, Frankfurt, Germany) and the HA tag (Santa Cruz Biotechnology, Heidelberg, Germany), according to each manufacturer's conditions. The antibody-bound proteins were detected using the ECL Plus system (Amersham Biosciences, Freiburg, Germany). Native protein extracts were prepared as described by Fan and Dong (2002).

### Copurification of NPR1 and NIMIN1

Copurification of NPR1 and NIMIN1 was done essentially as described by Fan and Dong (2002). The procedure was scaled down by a factor of 10.

### Yeast One-Hybrid System

The yeast assay for DNA binding of the ternary complex between NIMIN1, NPR1, and TGA factor was performed essentially as described by Weigel et al. (2001).

### RNA Gel Blot Analysis

Total RNA was extracted from 3-week-old plants, and 10 µg of each sample was used for RNA gel blot analysis as described previously (Weigel et al., 2001). Each time-course experiment was done in duplicate. Blots were stripped and reprobed two to three times. Fragments used to generate probes specific to *PR-1*, *PR-2*, *PR-5*, *ACTIN2*, and *NIMIN1* were amplified by PCR using primer combinations as described previously (Uknes et al., 1992; An et al., 1996; Weigel et al., 2001). RNA gel blot analysis was performed as described (Weigel et al., 2001).

### Infection with *Psm 4326* after Pretreatment with 1 mM SA

To generate macroscopic symptoms, 3-week-old plants of each genotype were used in three different treatments: one group was sprayed with 1 mM SA at 2 and 1 d before dipping in a bacterial suspension of *Psm 4326* (OD<sub>600</sub> = 0.2; in 10 mM MgCl<sub>2</sub> and 0.01% Silwett L-77; Lehle Seeds, Round Rock, TX). The second group was dipped in bacterial suspension without pretreatment with SA, and the third group was dipped in 10 mM MgCl<sub>2</sub> and 0.01% Silwett L-77. Plants were photographed at 4 DAI.

To assay the growth of bacteria in the plants, a combination of two methods was used (Tornero and Dangl, 2001; Katagiri et al., 2002). Plants were sprayed with 1 mM SA at 2 and 1 d before dipping in a bacterial suspension of *Psm 4326* (OD<sub>600</sub> = 0.002; in 10 mM MgCl<sub>2</sub> and 0.01% Silwett L-77). One hour after inoculation, the samples for day 0 were taken. Nine leaves were excised per genotype, and leaf discs were made from these samples using a hole puncher. Three sets of three discs per genotype were added to a preweighed 2-mL tube containing 1 mL of 10 mM MgCl<sub>2</sub> and 0.2% Silwett L-77. The tubes were then shaken on a vortex mixer set to 4 at room temperature for 10 min. After this time, 10 and 20 µL of each tube was spread onto a Petri dish of King's B medium (King et al., 1954) containing the appropriate antibiotics. For day 3, 12 leaves were excised per genotype and four sets of three leaf discs were used for the extraction of bacteria as described above. Extract (100 µL) was added to a microwell plate, and serial 10-fold dilutions were prepared using a multichannel pipette. The bacteria were spotted in 5-µL samples onto a 150-mm Petri plate of King's B medium containing the appropriate antibiotics using a multichannel pipette. Each plate was replicated once.

The plates were incubated for 2 d at 28°C. Dilutions that gave 1 to 60 colonies were counted. Each data point represents the mean and standard deviation of three independent experiments.

Infection with *Psm* 4326 *avrRpt2* was done essentially as described above but without pretreatment with 1 mM SA.

### Insertional Mutant Plant Isolation

A mutant carrying a T-DNA insertion in the *NIMIN1* gene was identified in an insertional mutant population (ecotype Col-0) (Sessions et al., 2002). Genomic DNAs were isolated using the method described by Weigel et al. (2001). The T-DNA insertion event in *NIMIN1* was confirmed by PCR and sequencing of the right and left border PCR products. Homozygous mutant plants for *nimin1-1* were identified by segregation analysis using the resistance marker kanamycin and RNA gel blot analysis. PCR analysis of plants carrying a homozygous insertion consistently yielded a single band using the combination of gene-specific and T-DNA border primers; however, as a result of the large insert size, no band was found using the two gene-specific primers.

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