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Strong Suppression of Systemic Acquired Resistance in *Arabidopsis* by NRR is Dependent on its Ability to Interact with NPR1and its Putative Repression Domain

Mawsheng Chern, Patrick E. Canlas and **Pamela C. Ronald**[1]

Department of Plant Pathology, University of California, Davis, CA 95616, USA

ABSTRACT Systemic Acquired Resistance (SAR) in plants confers lasting broad-spectrum resistance to pathogens and requires the phytohormone salicylic acid (SA). *Arabidopsis* NPR1/NIM1 is a key regulator of the SAR response. Studies attempting to reveal the function of NPR1 and how it mediates SA signaling have led to isolation of two classes of proteins that interact with NPR1: the first class includes rice NRR, *Arabidopsis* NIMIN1, NIMIN2, and NIMIN3, and tobacco NIMIN2-like proteins; the second class belongs to TGA transcription factors. We have previously shown that overexpression of NRR in rice suppresses both basal and *Xa21*-mediated resistance. In order to test whether NRR affects SA-induced, NPR1-mediated SAR, we have transformed *Arabidopsis* with the rice *NRR* gene and tested its effects on the defense response. Expression of *NRR* in *Arabidopsis* results in suppression of *PR* gene induction by SAR inducer and resistance to pathogens. These phenotypes are even more severe than those of the *npr1-1* mutant. The ability of NRR to suppress *PR* gene induction and disease resistance is correlated with its ability to bind to NPR1 because two point mutations in NRR, which reduce NPR1 binding, fail to suppress NPR1. In contrast, wild-type and a mutant NRR, which still binds to NPR1 strongly, retain the ability to suppress the SAR response. Replacing the C-terminal 79 amino acids of NRR with the VP16 activation domain turns the fusion protein into a transcriptional co-activator. These results indicate that NRR binds to NPR1 in vivo in a protein complex to inhibit transcriptional activation of *PR* genes and that NRR contains a transcription repression domain for active repression.

Key words: NRR; NPR1; SA; SAR; disease resistance.

INTRODUCTION

Systemic acquired resistance (SAR) is an induced defense response in plants; it induces expression of pathogenesis-related (*PR*) genes (Ryals et al., 1996) and confers lasting broad-spectrum resistance to viral, bacterial, and fungal pathogens. In dicots, such as *Arabidopsis* and tobacco, the phytohormone salicylic acid (SA) and the synthetic chemicals 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are potent inducers of SAR (Friedrich et al., 1996). The *NPR1* (for nonexpresser of *PR* genes 1; also known as *NIM1* and *SAI1*) gene is identified as a key regulator of the SA-mediated SAR pathway in *Arabidopsis* (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). *NPR1* expression levels become elevated upon induction by SA, INA, BTH, or pathogen infection (Cao et al., 1997; Ryals et al., 1997). *Arabidopsis npr1/nim1* mutants are impaired in their ability to induce *PR* gene expression and mount a SAR response, even after treatment with SA or INA.

Intensive investigations have shed some light on how NPR1 mediates SAR. NPR1 contains a bipartite nuclear localization sequence and two potential protein–protein interaction domains: an ankyrin repeat domain and a BTB/POZ domain (Cao et al., 1997; Ryals et al., 1997). NPR1 functions as a transcriptional co-activator in a TGA2–NPR1 complex after SA treatment in an in-vivo transient cell assay; this function requires the BTB/POZ domain and the oxidation of NPR1 Cys-521 and Cys-529 (Rochon et al., 2006). Nuclear localization of NPR1 protein is essential for its function (Kinkema et al., 2000). Without induction, NPR1 protein forms an oligomer

[1] To whom correspondence should be addressed. E-mail pcronald@ucdavis.edu, fax +1 530 752 5674.

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and is excluded from the nucleus. Redox changes mediate SAR induction, causing monomeric NPR1 to emerge and accumulate in the nucleus and activate *PR* gene expression (Mou et al. 2003).

In search of proteins that mediate NPR1 function, several groups have identified TGA family members of basic-region leucine zipper (bZIP) transcription factors, both from *Arabidopsis* (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000) and from rice (Chern et al., 2001), as NPR1 interacting proteins. The ankyrin repeats of NPR1 are necessary and sufficient for the interaction with TGA proteins but the interaction can be abolished by *npr1-1* and *npr1-2* mutants (Zhang et al., 1999). The interaction between NPR1 and TGA proteins facilitates in-vitro binding of the TGA proteins (Despres et al., 2000) and recruits them in vivo (Johnson et al., 2003) to the SA-responsive promoters. In-vivo interaction between NPR1 and a GAL4:TGA2 fusion (GAL4 DNA-binding domain fused to TGA2) protein leads to SA-mediated gene activation in *Arabidopsis* (Fan and Dong, 2002), supporting the notion that NPR1 binds in vivo to TGA2, which mediates transcriptional activation of downstream genes. The *Arabidopsis* triple knockout mutant *tga2tga5tga6* blocks induction of *PR* gene expression and pathogen resistance (Zhang et al., 2003), further supporting the hypothesis that TGA proteins mediate NPR1 function. TGA2, TGA5, and TGA6 function redundantly as negative regulators of *PR* genes before induction (Zhang et al., 2003; Rochon et al., 2006). It is thought that, after induction, TGA proteins serve to anchor NPR1 to *PR* gene promoters to activate the genes.

In *Arabidopsis*, another group of NIM1/NPR1 interacting proteins were identified and named NIMIN1, NIMIN2, and NIMIN3. These three *Arabidopsis* proteins share very limited sequence similarity but may be structurally related (Weigel et al., 2001). In tobacco, three NIMIN2-like (NIMIN2a, 2b, and 2c) were identified as NPR1 interactors (Zwicker et al., 2007). Weigel et al. (2005) further showed that overexpression of NIMIN1 in *Arabidopsis* led to abolishment of the SAR response after SA treatment and that knockout and RNA-silencing of *NIMIN1* resulted in enhanced *PR-1* gene expression after SA treatment, but no clear effects on disease resistance. In tobacco, Zwicker et al. (2007) showed that constitutive expression of *NtNIMIN2a* led to delayed PR-1 induction and suppression of *NIMIN2* transcripts enhanced the accumulation of PR-1 protein.

In *Arabidopsis*, overexpression of *NPR1* leads to enhanced disease resistance to both bacterial and oomycete pathogens (Cao et al., 1998; Friedrich et al., 2001). In rice, overexpression of *Arabidopsis NPR1* (Chern et al., 2001) or the rice homologue *NH1* (Chern et al., 2005b) results in enhanced resistance to the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), strongly suggesting the presence of a related defense pathway in rice.

We have previously reported the isolation and characterization of a novel rice gene *NRR* (for Negative Regulator of disease Resistance) (Chern et al., 2005a). Overexpression of *NRR* in rice leads to super-susceptibility to *Xoo*, impairing both basal and *Xa21*-mediated resistance. NRR interacts with both the *Arabidopsis* NPR1 protein and the rice NH1 protein. NRR shows limited similarity to the *Arabidopsis* and tobacco NIMIN2 proteins, only in the NPR1 interaction domain and a short EAR (ERF-associated amphiphilic repression; Ohta et al., 2001) motif-like sequence (LDLNxxP) near the C-terminus. Although function of tobacco NIMIN2a have been studied in transgenic tobacco, the roles of the *Arabidopsis* NIMIN2 and rice NRR in SAR have not been demonstrated. To probe the function of NRR, we transformed *Arabidopsis* with the *NRR* gene and tested effects on the SA-induced, NPR1-mediated SAR response. Here, we report striking suppression of the SAR response in *Arabidopsis* by rice NRR. These effects appear to be dependent on the ability of NRR to interact with NPR1 and its C-terminal half, containing the putative repression motif. Swapping the C-terminus of NRR with a VP16 transcription activation domain turns the protein into a transcriptional co-activator.

RESULTS

Expression of Rice *NRR* in *Arabidopsis* Results in Suppression of *PR* Gene Expression and Impaired Resistance to *Pseudomonas syringae*

The rice *NRR* gene was isolated based on interaction of its gene product with the *Arabidopsis* NPR1 protein in yeast (Chern et al., 2005a). We then tested whether NRR would affect NPR1-mediated SAR response in *Arabidopsis*, normally represented by induction of *PR* genes after treatment with SA or INA. We introduced a *35S-NRR* construct into wild-type *Arabidopsis* (carrying a *BG2-Gus* reporter gene (*BG2* = *PR-2*); Cao et al., 1994).

Many lines of 35S-NRR transgenic *Arabidopsis* were obtained and analyzed by a qualitative GUS histochemical staining assay. Most of them showed reduced GUS expression levels compared with the wild-type control after INA pretreatment (data not shown). Northern blot analysis was then used to assess RNA expression levels quantitatively. Figure 1 shows RNA expression levels from RNA blotting experiments of two (NRR-9 and NRR-12) of the *35S-NRR* lines along with wild-type (WT) and *npr1-1* controls. NRR-9 and NRR-12 show expression of *NRR* RNA, which is absent in WT and *npr1-1*. After treatment with INA (0.65 mM), *PR-1* and *PR-5* transcripts are highly induced whereas induction is impaired in *npr1-1*, except for the mild induction of *PR-5*. NRR-9 and NRR-12, like *npr1-1*, both failed to respond to INA induction. Interestingly, the levels of *PR-5* expression in NRR-9 and NRR-12 after induction are even lower than in the *npr1-1* mutant.

When these transgenic *Arabidopsis* plants were inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 at high concentration (10^6 cfu mL^{-1}) by syringe-infiltration, infected leaves of NRR-9 and NRR-12 collapsed in 2 d, as shown in Figure 2A. Figure 2A also shows that leaves of *npr1-1* partially collapsed while those of wild-type showed only mild speck symptoms.

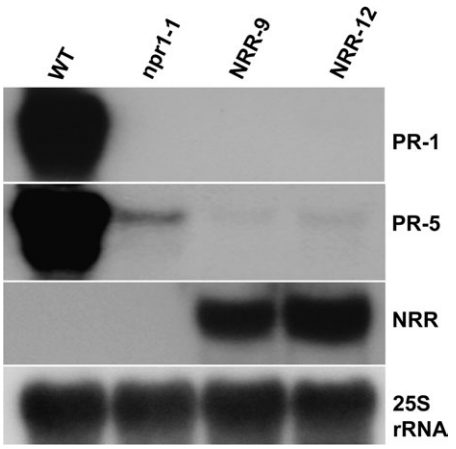

Figure 1. RNA Blot Analysis of *PR* Gene Expression after INA Induction.

Wild-type (WT) *Arabidopsis*, *npr1-1* mutant, and transgenic *Arabidopsis* (NRR-9 and NRR-12) carrying *35S-NRR* were treated with 0.65 mM INA. Two days later, tissues were collected and total RNA extracted from these samples. 10 micro grams of total RNA was loaded in each lane. The RNA blotted on a nitrocellulose membrane was probed with *PR-1*, *PR-5*, *NRR*, and 25S *rRNA* sequentially.

To test possible effects on SAR, these plants were sprayed with 0.65 mM INA to induce SAR and, 3 d afterwards, inoculated with *Pst* DC3000 at a lower concentration (10^5 cfu mL^{-1}) by syringe-infiltration. Leaf samples of these inoculated plants are shown in Figure 2B, where NRR-9 and NRR-12 displayed severe bacterial speck disease symptoms a week after inoculation. The *npr1-1* mutant only exhibited mild disease symptoms. On the contrary, the wild-type remained little affected. Figure 2C shows the growth curves conducted on these plants in the same experiment. For each sample, three leaves were pooled to extract *Pst* and the colony number was normalized to the weight of the leaves. The bacterial growth curves confirm that NRR-9 and NRR-12 are severely impaired in resistance to *Pst* DC3000 as they harbored several orders more *Pst* than the wild-type. The growth curves also confirmed that NRR-9 and NRR-12 are also more susceptible to *Pst* than the *npr1-1* mutant. *t*-tests give *p* values of 0.001 and 0.0001, respectively, for NRR-9 and NRR-12 compared with the *npr1-1* mutant, showing highly significant differences.

These results suggest that ectopically expressed NRR greatly suppresses normal defense responses, including SAR, in *Arabidopsis*, leading to hyper-susceptibility to *Pst* DC3000. Moreover, these plants were more severely diseased than the *npr1-1* mutant, which is impaired in SAR. Consistent with these observations are the data showing that NRR-9 and NRR-12 express lower levels of *PR-5* than *npr1-1* after INA induction.

NRR Mutants that Have Lost the Ability to Interact with NPR1 Lose the Ability to Suppress SAR in *Arabidopsis*

Previously, we have determined that the ability of NRR to interact with NPR1 depends on an NPR1-interacting (NI) domain comprising 25 amino acids (amino acids 28–52).

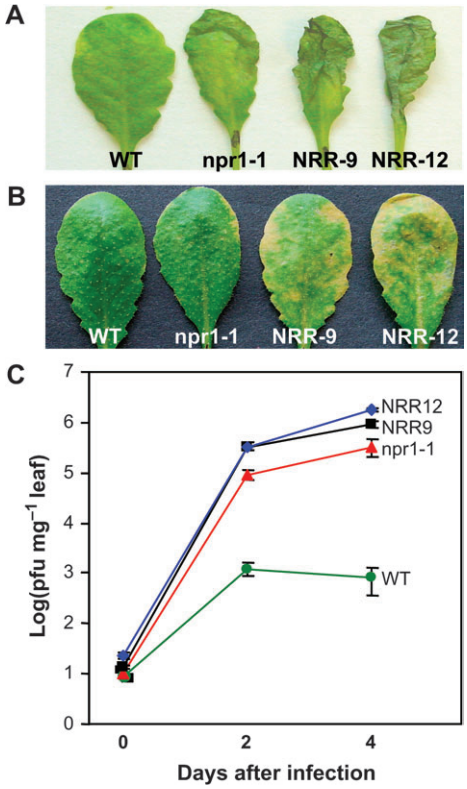

Figure 2. Symptoms of Infected *Arabidopsis* and Growth Curves of *P. syringae*.

(A) Leaf samples were taken 2 d after infection with *Pst* at a concentration 10^6 cfu mL^{-1}.

(B) *Arabidopsis* plants were infected with *Pst* at 10^5 cfu mL^{-1} 2 d after treatment with 0.65 mM INA. Leaf samples were taken 1 week after infection.

(C) Growth curves of *Pst*. *Arabidopsis* leaves were syringe-infiltrated with *Pst* at 10^5 cfu mL^{-1}. For each sample, three leaves were pooled to extract *Pst* and the colony number was normalized to the weight of the leaves. Each data point represents the average and standard deviation of four samples.

Figure 3A summarizes schematically the relative locations of the NI domain and three point mutations within it. As previously reported, two of the single point mutations—F40G (FG) and L44G (LG)—at amino acids conserved with NIMIN2 lead to the loss of most of the ability to interact with NPR1 in yeast, while E39K (EK) has little effect on interaction with NPR1 (Chern et al., 2005). To see whether the ability of NRR to suppress disease resistance is correlated with its ability to interact with NPR1, we transformed *Arabidopsis* with *NRR* mutants *FG* and *LG* under control of the *35S* promoter. For comparison, transgenic *Arabidopsis* carrying the wild-type *NRR* and mutant *EK* were also generated.

To assess the effects on expression of *PR* genes, we took advantage of the *BG2-Gus* reporter, in which the *Gus* gene is under control of an INA-responsive β-*1,3-glucanase* (*BG2*) promoter, in the recipient by assaying the GUS activity. After selection for the presence of the antibiotic resistance transformation marker, 18 independent T1 transgenic *Arabidopsis*

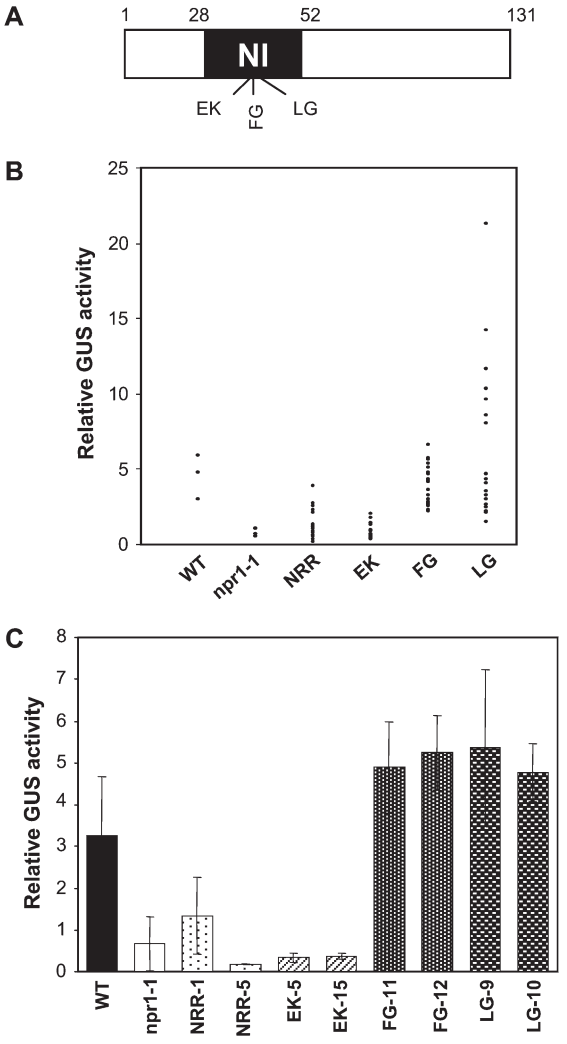

Figure 3. Gene Expression from the *BG2* Promoter after Induction.
(A) A schematic graph of the NRR protein depicting the locations of point mutations EK, FG, and LG.
(B) After selection on medium containing hygromycin, 18 independent T1 transgenic *Arabidopsis* carrying either wild-type NRR, EK, FG, or LG were transferred to plates containing INA to induce expression from the *BG2* promoter. GUS enzyme activity was assayed for each plant 3 d later. Three untransformed *BG2* (WT) and three *npr1-1* plants (in *BG2* background) were also assayed for comparison. Each dot represents the GUS activity of one plant.
(C) Progeny of two lines from each construct were selected for the GUS assay, along with the WT and *npr1-1* controls. Five plants of each line were assayed individually after INA induction. Each bar represents the average and standard deviation of five plants.

carrying either wild-type *NRR*, *EK*, *FG*, or *LG* transgene were transferred to plates containing INA to induce expression from the *BG2* promoter. Quantitative GUS enzyme activity was assayed for each plant 3 d afterwards. For comparison, GUS activity of three untransformed *BG2* (WT) and three *npr1-1* plants (in the *BG2* background) were assayed.

The GUS activity data were plotted in Figure 3B, where each dot represents one plant. Consistent with previous observations, the GUS activities of the majority of NRR plants shifted downward towards that of *npr1-1*. Plants of the EK mutants responded like the NRR plants. The FG plants responded similarly to WT. The LG plants also behaved similarly to WT, despite having a much broader distribution. To confirm these results, progeny of two lines from each construct were selected for the GUS assay. Five plants of each line were assayed individually after INA induction and the results are shown in Figure 3C. Figure 3C confirms the observations that NRR and EK transgenic *Arabidopsis* plants behave like *npr1-1* whereas FG and LG transgenic plants responded similarly to wild-type *Arabidopsis*. Together, these data suggest that while NRR and EK suppress *PR* gene induction, FG and LG, which lost the ability to interact strongly with NPR1, lose the suppression. Therefore, the ability of NRR to suppress *PR* gene induction is correlated with its ability to interact with NPR1.

These transgenic *Arabidopsis* plants were also tested for response to challenge by *Pst* DC3000. They were syringe-infiltrated with *Pst* DC3000 at a concentration of 10^5 cfu mL^{-1} after treatment with INA. Figure 4A shows the bacterial growth curves in the different transgenic plants. Consistent with the effects on *PR* genes, the FG and LG mutants behaved similarly to wild-type while NRR and EK responded like *npr1-1*. Figure 4B shows a typical leaf from each plant 7 d after inoculation. Similarly, NRR and EK plants showed severe speck disease and *npr1-1* was mildly diseased. On the contrary, the FG and LG plants, like wild-type, showed few signs of the disease.

To confirm that the protein was expressed in the individual transgenic plant, immunoblot analysis was performed. Protein extracted from the lines was blotted on a nitrocellulose membrane and probed with antibodies raised against a peptide corresponding to the C-terminal half of NRR. Figure 4C shows that the mutant FG and LG proteins are at least as stable as the wild-type NRR and EK proteins. Thus, loss of the suppression effects in FG and LG cannot be accounted for by protein instability.

A Domain Swap Turns NRR into a Transcriptional Activator that Enhances Disease Resistance

The observation that NRR suppresses induction of *PR* gene suggests that it may contain a repression domain and act as a transcriptional repressor when bound to NPR1. One way to test this is to swap out the possible repression domain in NRR. NRR is a small protein made of 131 amino acids. Its N-terminal half contains a nuclear localization signal and the NPR1-interacting domain; its C-terminal half is proline- and alanine-rich and contains an LDLNxxP sequence, resembling the ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001; Chern et al., 2005a), near the C-terminal end. To swap out the putative repression domain, the N-terminal first 52 amino acids of NRR was fused to a VP16 activation domain (replacing the C-terminal 79 amino acids), resulting in a fusion protein NIAD. The ability of the NIAD fusion protein to bind to NPR1 strongly was demonstrated previously in yeast two-hybrid (Chern et al., 2005).

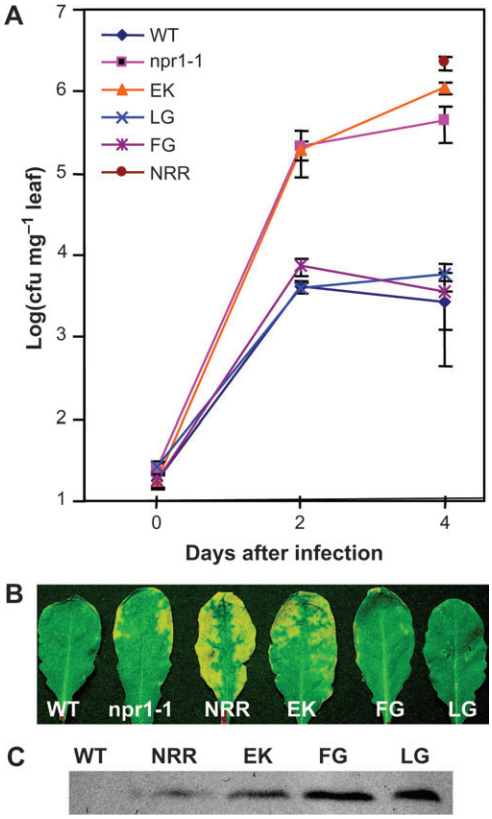

Figure 4. Growth Curves of *P. syringae* and Bacterial Speck Symptoms of *Arabidopsis* Carrying Wild-Type NRR and NRR Mutants.

(A) Growth curves of *Pst*. The inoculation and growth curves were done the same way as described in Figure 2B and 2C.

(B) Bacterial speck symptoms of WT *Arabidopsis*, *npr1-1*, and *Arabidopsis* carrying wild-type *NRR*, mutants *EK*, *FG*, and *LG*, after infection with *Pst*.

(C) Western blot analysis. Equal amounts (200 micro grams) of protein extracted from WT *Arabidopsis*, *Arabidopsis* carrying wild-type NRR, mutants EK, FG, and LG were loaded, run on a gel, and blotted to a nitrocellulose membrane. The membrane was probed with an antiserum raised against the C-terminal 83 amino acids of NRR, excluding the NI domain, where the three mutations are located.

The *NIAD* construct was transformed into *Arabidopsis* under control of a maize ubiquitin promoter (Christensen and Quail, 1996). Progeny of five lines were assayed for GUS activity from the *BG2-Gus* reporter gene after 0.1 mM INA induction of *PR* gene expression. Figure 5A shows the GUS activity of the average of three plants. Lines NIAD-10 and NIAD-12 showed much higher GUS activity than the wild-type. NIAD-22 showed only slightly higher GUS activity in Figure 5A, but, when the assay was repeated with more plants, it exhibited a two-fold higher GUS activity than WT (data not shown).

These three NIAD lines (NIAD-10, NIAD-12, and NIAD-22) along with the wild-type control were challenged with *Pst* DC3000 1 d after induction by INA. We chose to spread the plants with 0.1 mM INA instead of 0.65 mM INA because 0.65 mM INA strongly induces SAR and can easily mask any possible enhanced resistance responses. Figure 5B shows the

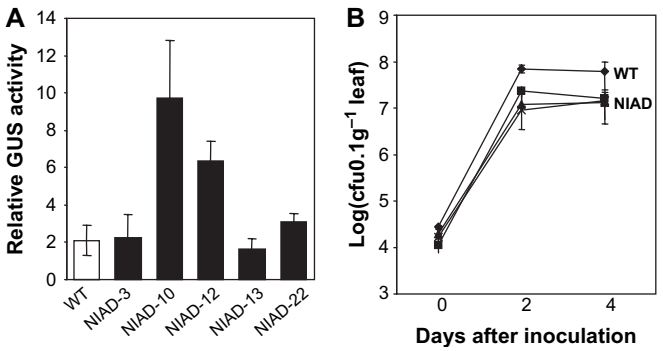

Figure 5. *PR* Gene Expression after Induction and *P. syringae* Growth Curves in NIAD Plants.

(A) GUS activity driven by the *BG2* promoter after INA induction. Along with the WT, five lines of NIAD transgenic *Arabidopsis* were assayed for GUS activity expressed from the *BG2-Gus* reporter gene after INA induction to induce *PR* gene expression. Each bar represents the average and standard deviation of three plants.

(B) Growth curves of *Pst*. *Arabidopsis* plants were sprayed with 0.1 mM INA 24 h before syringe-infiltration with *Pst* at 10^5 cfu mL^{-1}. Growth curves were done similarly as described in Figure 2C. Each data point represents the average and standard deviation of three samples.

bacterial growth curves in these plants, where each data point represents three repeats. The bacterial populations in all three NIAD lines are lower than in the wild-type. At day 4 after inoculation, the average bacterial populations in the wild-type are approximately four- to five-fold larger than those in the three NIAD lines. When the nine data points of all three NIAD lines are taken into consideration, the *t*-test gives a *p* value of 0.0023, demonstrating a highly significant difference between NIAD and the wild-type control. Together, these results show that expression of the NIAD fusion protein enhances induction of *PR* gene expression after INA treatment, leading to higher resistance to *Pst* DC3000. These data also indicate that the NIAD fusion protein functions as a transcriptional activator when bound to NPR1 in *Arabidopsis*.

DISCUSSION

We have shown that expression of the rice NRR in *Arabidopsis* results in severe suppression of the SAR response. This suppression is dependent on the ability to interact with the NPR1 protein. These results suggest that NRR binds directly to NPR1 in vivo to inhibit the function of NPR1. *Arabidopsis* NPR1 is thought to act as a transcriptional co-activator as demonstrated in a transient assay system (Rochon et al., 2006). NRR contains an EAR-like motif (LDLNxxP) near its C-terminus (Chern et al., 2005a). Thus, NRR most likely acts as a transcriptional repressor by active repression. Indeed, replacing the C-terminal 79 amino acids of NRR with a VP16 activation domain turns the fusion protein NIAD into a co-activator. Expression of the fusion protein NIAD in *Arabidopsis* leads to stronger activation of the *BG2* (*PR-2*) promoter, which is representative of *PR* genes, after INA induction of the SAR response. An unlikely

scenario remains that the higher *BG2-GUS* expression and resistance to *P. syringae* results from squelching by an overexpressed transcriptional activation domain, namely the VP16 activation domain. Our data strongly suggest that NIAD is present in a complex with NPR1 and TGA that binds to and activates the *BG2* promoter. It therefore indicates that NRR probably forms a complex with NPR1 and TGA in vivo to act as a transcriptional repressor.

It is interesting that expression of *NRR* in *Arabidopsis* results in suppression of the INA-induced SAR response, including activation of the *PR-5* gene and resistance to *Pst*, even more severe than the *npr1-1* mutation does. Weigel et al. (2005) showed that constitutive expression of *Arabidopsis* NIMIN1 in *Arabidopsis* led to an *npr1-1* like phenotype, but no phenotypes more severe than *npr1-1* were reported. These results indicate that there are differences between rice NRR and *Arabidopsis* NIMIN1 despite their ability to bind to NPR1. Rice NRR may carry a more potent transcriptional repression domain, which may be lacking in *Arabidopsis* NIMIN1. How does constitutive expression of NRR suppress SAR more severely than the *npr1-1* mutant? It could be because the *npr1-1* mutation still carries some residual NPR1 activity while NRR completely inhibits NPR1 function to induce *PR* gene expression. Another possible scenario is that NRR may also inhibit components other than NPR1. It is known that there are SA-dependent but NPR1-independent pathways leading to activation of *PR* genes. For example, the *Arabidopsis ssi* mutation causes accumulation of SA, leading to constitutive expression of *PR-1*, *PR-2*, and *PR-5* in the *npr1-5* background (Shah et al., 1999). In another case, sucrose is shown to increase *PR-2* and *PR-5* gene expression through an SA-dependent but NPR1-independent pathway (Thibaud et al., 2004). NRR may inhibit one of the components involved in one of these NPR1-independent pathways.

It is conceivable that NRR may interact with *Arabidopsis* NPR1 paralogs, such as NPR2, NPR3, and NPR4, since NRR interacts with paralogs of NH1 (rice NPR1 homolog 1) in rice (Chern, M. unpublished). However, since *Arabidopsis* NPR3 and NPR4 are shown to negatively regulate both NPR1-dependent and NPR1-independent pathways and *PR* gene expression (Zhang et al., 2006), NRR is not likely to inhibit NPR3 and NPR4 directly. No biological function of the *Arabidopsis NPR2* gene has been reported so far. It remains possible that NPR2 may have partial overlapping function with NPR1 in mediating the SA signaling pathway and that it may mediate partial activation of *PR* gene expression when NPR1 is no longer functional. It is possible that NRR interacts with *Arabidopsis* NPR2 and interferes with NPR2 function.

METHODS

Generation of Mutant Constructs and Transgenic *Arabidopsis*

A 35S–C1300 vector was first created by cloning the *CaMV 35S* promoter and a nos 3′ terminator sequence into the pCambia 1300 vector. Wild-type *NRR* and mutant genes *EK*, *FG*, and *LG* were then cloned into the *Bam*HI site behind the *35S* promoter to generate *35S-NRR*/C1300, *35-EK*/C1300, *35S-FG*/C1300, and *35S-LG*/C1300. The *Ubi–NIAD* construct was created by cloning the *NIAD* gene chimera (a *Bam*HI/*Kpn*I fragment) into the *Bam*HI and *Kpn*I sites in the Ubi–C1300 vector. Generation of NRR single point mutants E39K, F40G, L44G, and chimera NIAD containing the first 52 amino acids (NI) and VP16 activation domain (AD) has been described before (Chern et al., 2005a).

To generate transgenic *Arabidopsis*, each of the constructs described above was used to transform Agrobaterium EHA105, which was then used to transform *Arabidopsis thaliana* Col using a dipping method (Bechtold et al., 1993). *Arabidopsis* seeds were germinated on MS (Murashige and Skoog) medium containing 50 mg L^{-1} hygromycin for selection.

SAR Induction by INA Treatment

For quantitative GUS activity measurement, the plants were grown for several days on MS basal medium agar plates containing 0.1 mM INA. The plant was ground up in GUS buffer and GUS enzyme activity assay was performed as described by Jefferson (1987). For *P. syringae* disease testing and RNA extraction, plants were grown on soil for several weeks and 0.1 or 0.65 mM INA was spread by foliar application 1–3 d before the assays.

RNA Blot Analysis and *P. syringae* Disease Testing

Total RNA was extracted from harvested tissue using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was run on denaturizing agarose gels containing formaldehyde. The RNA was then transferred to a nitrocellulose membrane and hybridized to different probes. *P. syringae* was re-suspended in 10 mM $MgSO_4$ solution at the desired concentration and infiltrated into each leaf by a 1-mL syringe. For *P. syringae* growth curves, three leaves were collected for each data point and ground up in 10 mM $MgSO_4$ solution to extract the bacteria. Bacteria were plated out at a series of dilution on NYGA (0.5% peptone, 0.3% yeast extract, 2% glycerol, and 1.5% agar) medium containing rifampicin (25 mg L^{-1}).

Generation of Polyclonal Antibodies against NRR

A DNA fragment corresponding to the C-terminal 83 amino acids of NRR (NRRC) was PCR-amplified with primers NRRC-pET (TTTCATATGGACGCCACCCGACGGCTC) and mn45-4 (AGGATCCACTAGTCTCGAGTTGTAATCCGTGAGCA). The PCR product was purified and digested with NdeI + BamHI and cloned in-frame into vector pET15b, pre-digested with *Nde*I and *Bam*HI. The NRRC peptide was expressed in *E. coli* BL21(DE3)pLysS cells and purified with Ni-NTA agarose resins, as described before. The purified NRRC peptide was used to inject rabbits to raise polyclonal antisera. The antisera were tested against *E. coli* protein extracts with and without NRR to confirm specificity.

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REFERENCES

Bechtold, N., Ellis, J., and Pelletier, G. (1993). In planta Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C.R. Acad. Sci. Paris, Life Sciences **316,** 1194–1199.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583–1592.

Cao, H., Glazebrook, J., Clarke, J., Volko, S., and Dong, X. (1997). The Arabidopsis npr1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57–63.

Cao, H., Li, X., and Dong, X. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl Acad. Sci. U S A **95,** 6531–6536.

Chern, M., Canlas, P.E., Fitzgerald, H.A., and Ronald, P.C. (2005a). Rice NRR, a negative regulator of disease resistance, interacts with Arabidopsis NPR1 and rice NH1. Plant J **43,** 623–635.

Chern, M., Fitzgerald, H.A., Canlas, P.E., Navarre, D.A., and Ronald, P.C. (2005b). Over-expression of a rice NPR1 homologue leads to constitutive activation of defense response and hypersensitivity to light. Mol. Plant–Microbe Interact **18,** 511–520.

Chern, M.-S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X., and Ronald, P.C. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. Plant J **27,** 101–113.

Christensen, A.H., and Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res **5,** 213–218.

Delaney, T.P., Friedrich, L., and Ryals, J.A. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl Acad. Sci. U S A **92,** 6602–6606.

Despres, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. (2000). The Arabidopsis npr1/nim1 protein enhances the DNA binding activity of a subgroup of the TGAfamily of bZIP transcription factors. Plant Cell **12,** 279–290.

Fan, W., and Dong, X. (2002). *In vivo* interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14,** 1377–1389.

Friedrich, L., et al. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J **9,** 61–70.

Friedrich, L., Lawton, K., Dietrich, R., Willitis, M., Cade, R., and Ryals, J. (2001). NIM1 overexpression in Arabidopsis potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Mol. Plant–Microbe Interact **9,** 1114–1124.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973–982.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep **5,** 387–405.

Johnson, C., Boden, E., and Arias, J. (2003). Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in Arabidopsis. Plant Cell **15,** 1846–1858.

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 isrequired for activation of PR gene expression. Plant Cell **12,** 2339–2350.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113,** 1–10.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressor share an essential motif for active repression. Plant Cell **13,** 1959–1968.

Ryals, J., et al. (1997). The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell **9,** 425–439.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8,** 1809–1819.

Shah, J., Kachroo, P., and Klessig, D.F. (1999). The Arabidopsis *ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. Plant Cell **11,** 191–206.

Shah, J., Tsui, F., and Klessig, D.F. (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Mol. Plant Microbe Interact **10,** 69–78.

Thibaud, M.-C., Gineste, S., Nussaume, L., and Robaglia, C. (2004). Sucrose increases pathogenesis-related PR-2 gene expression in Arabidopsis thaliana through an SA-dependent but NPR1-independent signaling pathways. Plant Physio. Biochem **42,** 81–88.

Weigel, R.R., Bauscher, C., Pfitzner, A.J., and Pfitzner, U.M. (2001). NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. Plant Mol. Biol **46,** 143–160.

Weigel, R.R., Pfitzner, U.M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in Arabidopsis. Plant Cell **17,** 1279–1291.

Zhang, Y., Cheng, Y.T., Qu, N., Zhao, Q., Bi, D., and Li, X. (2006). Negative regulation of defense responses in Arabidopsis by two NPR1 paralogs. Plant J **48,** 647–656.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc. Natl Acad. Sci. U S A **96,** 6523–6528.

Zhang, Y., Tessaro, M.J., Lassner, M., and Li, X. (2003). Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15,** 2647–2653.

Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol. Plant–Microbe Interact **13,** 191–202.

Zwicker, S., Mast, S., Stos, V., Pfitzner, A.J.P., and Pfitzner, U.M. (2007). Tobacco NIMIN2 proteins control *PR* gene induction through transient repression early in systemic acquired resistance. Mol. Plant Pathol **8,** 385–400.