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Rice NRR, a negative regulator of disease resistance, interacts with Arabidopsis NPR1 and rice NH1

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Summary

Arabidopsis NPR1/NIM1 is a key regulator of systemic acquired resistance (SAR), which confers lasting broad-spectrum resistance. Over-expression of Arabidopsis NPR1 or the NPR1 homolog 1 (NH1) in rice results in enhanced resistance to the pathogen Xanthomonas oryzae pv. oryzae (Xoo), suggesting the presence of a related defense pathway in rice. We investigated this pathway in rice by identifying proteins that interact with NH1. Here we report the isolation and characterization of a rice cDNA encoding a novel protein, named NRR (for negative regulator of resistance). NRR interacts with NPR1 in the NPR1-interacting domain (NI25) consisting of 25 amino acids. NRR also interacts with NH1; however, NI25 was not sufficient for a strong interaction, indicating a difference between the rice and the Arabidopsis proteins. Silencing of NRR in rice had little effect on resistance to Xoo. When constitutively over-expressed in rice, NRR affected basal resistance, age-related resistance and Xa21-mediated resistance, causing enhanced susceptibility to Xoo. This phenotype was correlated with elevated NRR mRNA and protein levels and increased Xoo growth. Over-expression of NRR suppressed the induction of defense-related genes. NRR:GFP (green fluorescent protein) protein was localized to the nucleus, indicating that NRR may act directly to suppress the activation of defense genes. The fact that NRR compromises Xa21-mediated resistance indicates cross-talk or overlap between NH1- and Xa21-mediated pathways.

Keywords: rice, NRR, NH1, NPR1, Xa21, disease resistance.

Introduction

Systemic acquired resistance (SAR) is an important defense response in plants. SAR induces the 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) as well as the synthetic chemicals 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are potent inducers of SAR (Friedrich et al., 1996). In monocots, SAR was shown to be induced by BTH in wheat (Gorlach et al., 1996) and by Pseudomonas syringae in rice (Smith and Metraux, 1991). BTH can also induce disease resistance in rice (Rohilla et al., 2002; Schweizer et al., 1999) and maize (Morris et al., 1998), although it is not clear whether the resistance was SAR. The NPR1 gene (also known as NIM1 and SAI1) is 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). Upon induction by SA, INA or BTH, NPR1 expression levels are elevated (Cao et al., 1997; Ryals et al., 1997). NPR1 affects the SAR pathway downstream of the SA signal. 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.

Over-expression of NPR1 in Arabidopsis leads to enhanced disease resistance to both bacterial and oomycete pathogens (Cao et al., 1998; Friedrich et al., 2001). Similarly, over-expression of Arabidopsis NPR1 (Chern et al., 2001) and the homolog NH1 (Chern et al., 2005) in rice results in enhanced resistance to the pathogen Xanthomonas oryzae pv. oryzae (Xoo), suggesting the presence of a related defense pathway in rice. However, there are differences in Arabidopsis and rice with regard to their response to the elevated levels of NPR1 and NH1. Although transgenic Arabidopsis plants over-expressing NPR1 acquire enhanced
sensitivity to SA and BTH (Friedrich et al., 2001), they display no obvious detrimental morphological changes and mostly do not have elevated PR gene expression until activated by inducers or by infection of pathogens (Cao et al., 1998; Friedrich et al., 2001). However, in rice over-expression of NH1 leads to constitutive activation of defense genes and causes a developmentally controlled lesion mimic phenotype (Chern et al., 2005). In addition, over-expression of either NH1 or NPR1 in rice potentiates a lesion mimic/cell death (LMD) phenotype that can be triggered by BTH treatment or low light intensity (Fitzgerald et al., 2004).

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). Nuclear localization of NPR1 protein is essential for its function (Kinkema et al., 2000). Without induction, NPR1 protein forms an oligomer and is excluded from the nucleus. Redox changes mediate induction of SAR, causing monomeric NPR1 to emerge and accumulate in the nucleus and activate PR gene expression (Mou et al., 2003).

In the search for proteins that mediate NPR1 function, several groups have identified TGA family members of basic-region leucine zipper (bZIP) transcription factors, either from Arabidopsis (Despres et al., 2000; Zhang et al., 1999; Zhou et al., 2000) or 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 vivo 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 protein (GAL4 DNA-binding domain fused to TGA2) leads to SA-mediated gene activation in Arabidopsis (Fan and Dong, 2002). The essential role of TGA proteins in the SAR response was clearly demonstrated by analyzing the triple-knockout mutant tga2tga5tga6, which blocked induction of PR gene expression and pathogen resistance (Zhang et al., 2003). In short, it has become clear that TGA proteins serve as a bridge between NPR1 and PR gene induction.

In Arabidopsis, another group of NIM1/NPR1-interacting proteins, named NIMIN1, -2 and -3, were identified. These three Arabidopsis proteins share very limited sequence similarity but may be structurally related (Weigel et al., 2001). Modulation of PR gene expression in Arabidopsis by interaction of NIMIN1 with NPR1 has recently been reported (Weigel et al., 2005).

Rice, being the most important food crop for human consumption, has emerged as the model system for monocots, especially cereals. Although rice has attracted great research interest upon the completion of it genome sequence, relatively little is known about the pathways and mechanisms leading to disease resistance, including the NH1-mediated pathway. Rice is different from tobacco and Arabidopsis in that it has very high basal levels of SA, and no changes in SA levels were detected after interactions with avirulent or virulent pathogens (Silverman et al., 1995). Thus, in rice pathways and mechanisms leading to resistance and their regulation may be quite different from those in Arabidopsis. To further characterize the NH1-mediated pathway in rice, we have identified rice cDNA clones encoding proteins that interact with NH1 and NPR1. Here we report the isolation and characterization of one of the rice cDNA clones called NRR. Transgenic rice plants constitutively over-expressing NRR display multiple phenotypic changes, including altered basal resistance, age-related resistance and Xa21-mediated resistance to Xoo.

Results

Isolation of NRR from rice and similarity to other proteins

We have previously reported the isolation of four different families of NPR1-interacting proteins after screening approximately 20 million yeast colonies transformed with a rice cDNA library (Chern et al., 2001). The first group contains four members belonging to the bZIP family of transcription factors (Chern et al., 2001). Here we report on the second group of NPR1-interacting proteins that contains a single member, named NRR (for negative regulator of resistance). NRR cDNA clones of different lengths were isolated more than 20 times from yeast two-hybrid screens. The full-length NRR protein is predicted to encode 131 amino acids (accession no. AY846391). RNA blot analysis showed that the NRR mRNA is approximately 800 nucleotides (nt) (data not shown), approximately the size of the cDNA. Comparison of NRR cDNA and genomic sequences revealed that the NRR gene contains no intron.

The NRR protein shows no significant homology to known protein domains. However, the C-terminal half of NRR is proline rich (30% proline). Blast searches of the GenBank databases identified at least three proteins that are similar to NRR. Figure 1 presents the amino acid alignment of rice NRR, the wheat NRR homolog (wNRR) encoded by an expressed sequence tag (EST) clone (gi: 5799981), Arabidopsis NIMIN2 and tobacco G8-1 proteins. Arabidopsis NIMIN2 was identified as a NIM1/NPR1-interacting protein (Weigel et al., 2001); G8-1 was identified as an SA-inducible gene that showed rapid mRNA accumulation after treatment with SA (Horvath et al., 1998). NRR and wNRR are highly similar across the whole protein. NRR, NIMIN2, and G8-1 share very limited similarity, mainly in a small region (highlighted in Figure 1) containing about 20 amino acids. All four proteins contain the sequence LDLNxxP (aligned and highlighted in bold in Figure 1) resembling the EAR
(ERF-associated amphiphilic repression) motif, which was identified as an active transcription repression domain in the family of class II ERF transcription factors (Ohta et al., 2001).

In addition, NRR, wNRR, NIMIN2 and G8-1 all contain a putative nuclear localization signal sequence KRKR or KKRKR (in bold italic style in Figure 1). Thus, NRR, wNRR, NIMIN2 and G8-1 appear to be structurally related.

Interaction of NRR with Arabidopsis NPR1 and rice NH1 proteins requires different domains in the NRR protein

NRR was further characterized in the yeast two-hybrid system by deletion and point mutation analysis to localize the region and amino acids required for interaction with NPR1 and NH1. In the left panel of Figure 2(a), the full-length NRR (labeled NRR), fused to the B42 activation domain in the pB42AD vector (Clontech, Mountain View, CA, USA), interacted strongly with NPR1 (demonstrated by the dark blue color) while the empty vector did not interact with NPR1. A region containing 25 amino acids (nos 28–52, named NI25; marked by a hatched bar above in Figure 1) in NRR, covering the region showing sequence similarity to NIMIN2 and G8-1, was capable of interacting with NPR1. Three point mutations (EK, FG and LG; marked by arrowheads in Figure 1) located in this region at amino acids 39, 40 and 44, respectively, were generated. Mutations FG and LG almost completely abolished interaction with NPR1. The effects of the FG and LG mutations on interaction with NPR1 were not due to protein instability, because both FG and LG proteins accumulated abundantly in yeast (Figure 2a, right panel). Mutation EK had little effect on β-galactosidase reporter activity. However, because the EK protein was much more abundant than the wild-type protein in yeast cells, subtle effects of EK on interaction with NPR1 cannot be ruled out. The B42:NI25 protein was detected, though at a lower level. The B42:NRR fusion protein was present at a very low level in yeast since it was not detected by antibodies against the hemagglutinin (HA)-tag on the fusion protein. These experiments not only show the interaction between NRR and NPR1 but also identify a short peptide (NI25) sufficient for interaction with NPR1 and two amino acids (F40 and L44) important for the interaction.

Interaction with the rice NH1, which shares 49% identity with NPR1 (Chern et al., 2005), was also tested in the system. As shown in Figure 2(b) (left panel), the full-length NRR interacts strongly with rice NH1 while the vector control shows no interaction. Surprisingly, NI25 was not sufficient for interaction with NH1. The FG and LG mutations only had minor effects on the interaction. These results suggest that the strong interactions of NRR with NH1 and with NPR1 are not identical because NRR requires additional regions to interact with NH1. Specific interaction between NH1 and wild-type NRR was confirmed by immuno-coprecipitation of yeast-expressed LexA:NH1 and B42AD:NRR. The B42AD:NRR fusion protein was pulled down by a monoclonal antibody against the HA-tag at the C-terminal of B42AD. The co-precipitated LexA:NH1 protein was detected by a monoclonal antibody against the LexA DNA-binding domain. Figure 2(c) shows that the LexA:NH1 protein was co-precipitated by B42AD:NRR (labeled NRR) but not by B42AD (labeled vector).

We created two additional fusion constructs containing the first 52 amino acids (NRR52) and the first 76 amino acids (NRR76) of NRR, respectively. NRR52, containing all the N-terminal amino acids up to NI25, interacts with NH1 only weakly (Figure 2b, right panel). In contrast, NRR76 interacts with NH1 as strongly as the full-length NRR. Thus, the region between amino acids 52 and 76 is required for strong interaction with rice NH1. This region shares homology with wNRR but not with NIMIN2 or G8-1, and thus may be unique to monocot proteins.

Silencing of NRR has little effect on resistance to Xoo

To study the possible role of NRR in regulating rice defense, we silenced or constitutively over-expressed the NRR gene.
First, we generated at least 20 transgenic rice lines (called NRRsi) carrying an RNA interference (double-stranded RNA) construct to silence the NRR gene in the Liaogeng (LG) rice cultivar, which is moderately susceptible to Xoo Philippine race 6 (PR6, strain POX99). Twenty putative NRRsi lines were inoculated with Xoo PR6 to assess the effects on resistance. Figure 3(a) shows the inoculation results. No clear effects on resistance were observed, despite some slight deviations from the LG control; these deviations were not statistically significant and are within the range of typically observed biological variations. Segregating T₁ progeny of lines 41 (slightly more resistant) and 72 (slightly more susceptible) were inoculated with Xoo PR6 to determine if the phenotypes in T₀ were heritable. The progeny displayed the same phenotype as the LG control and no segregation of resistance was observed (see Figure S1).

We carried out RNA blot analysis to determine if the NRRsi lines had reduced NRR gene expression. RNA samples from seven of the NRRsi lines plus the LG control were hybridized with an NRR probe and then to a 25S rRNA probe. Figure 3(b) shows that NRR is only expressed at a low level in the wild-type LG cultivar, whereas six of the seven lines of the NRRsi transgenic lines had lower NRR mRNA levels, indicating that silencing was effective in all lines except line 73. Hybridization with PR-10 and POX22.3 probes showed no differences in expression between LG and NRRsi lines (data not shown).

Constitutive over-expression of NRR in rice affects both basal resistance and age-related resistance to Xoo, causing enhanced susceptibility

We used a maize ubiquitin promoter (Christensen and Quail, 1996) to constitutively over-express the NRR cDNA in rice. We first transformed this Ubi–NRR construct into the rice...
cultivar Taipei 309 (TP309), which is susceptible to Xoo PR6. At least eight independently transformed lines (ITLs) carrying the Ubi–NRR transgene (called UNRR lines), resistant to hygromycin selection, were generated in this experiment. Transgenic plants were inoculated at 6 weeks of age along with the TP309 control and lesion lengths were measured 2 weeks later. Figure 4(a) displays the lengths of leaf lesions in seven UNRR ITLs after inoculation. All UNRR lines except line 2 showed longer lesions than the control. Strikingly, line 8 died approximately 4 weeks after the inoculation; some of the inoculated tillers of lines 10 and 11 also died. These results indicate that over-expression of NRR may affect basal levels of resistance to Xoo in rice. Obtaining fertile rice seeds from these lines proved difficult; we therefore switched to LG as the recipient for our rice transformation studies because LG has better seed set.

We obtained more than 17 hygromycin-resistant UNRR ITLs in the LG background. Figure 4(b) shows results for lesion length collected 2 weeks after inoculation with Xoo PR6. Twelve UNRR ITLs were inoculated at 6 weeks after regeneration; most UNRR lines developed lesions with lengths longer than the LG control, reflecting an enhanced susceptibility phenotype. These results further support the conclusion that over-expression of NRR affects basal levels of resistance to Xoo in rice. The LG control and five UNRR lines were inoculated 11 weeks after regeneration. The LG control acquired high levels of resistance at 11 weeks of age; all five UNRR lines showed longer lesion lengths. These results suggest that over-expression of NRR may affect the age-related resistance acquired by older rice plants.

To study whether the enhanced susceptibility phenotype is caused by the Ubi–NRR transgene, segregating progeny from lines 64 (UNRR-64) and 67 (UNRR-67) were analyzed. PCR tests specific to the Ubi–NRR transgene were performed with one primer annealing to the ubiquitin promoter and the other to the NRR cDNA. Progeny carrying the Ubi–NRR transgene are labeled UNRR (in green) and null segregants that no longer carry the transgene are labeled UNRR– (in blue) in Figure 5. Plants were then inoculated at approximately 10 weeks of age. Figure 5(a) shows the lengths of the lesions in these segregating progeny and in the LG control in a bar graph. Figure 5(b) shows a picture of three typical leaves from each of the LG, UNRR and UNRR– groups. The UNRR progeny all showed longer lesions while the UNRR– progeny displayed lesions with lengths similar to the LG control. Thus the enhanced susceptibility phenotype correlated with the presence of the Ubi–NRR transgene.

Leaves from each group were collected separately after measurement of the lesion length. Each leaf was ground up to measure the Xoo population. It should be noted that the leaf sizes of UNRR progeny were similar to those of the LG control and UNRR– progeny. Figure 5(c) shows that the UNRR (in green) progeny from both lines 64 and 67 sustained Xoo populations which were approximately 10 times higher than the UNRR– (in blue) progeny, which carried similar numbers of Xoo as the LG control. These results support the results from measurements of lesion length and show that over-expression of NRR affects age-related resistance to Xoo, causing enhanced susceptibility.

Over-expression of NRR compromises Xa21-mediated resistance to Xoo

The rice Xa21 disease resistance gene, encoding a leucine-rich repeat (LRR) receptor-like protein kinase (RLK; Song et al., 1995), confers robust resistance to many isolates of the Xoo pathogen, including isolate PR6 PXO99. The results observed above prompted us to investigate if constitutive over-expression of NRR would also affect Xa21-conferred resistance. A transgenic rice line carrying the Xa21 gene was available in the TP309 (a japonica cultivar) background (Song et al., 1995). This line was used as the recipient for
transformation with the *Ubi–NRR* gene; the *Bar* gene selection was utilized because the *Xa21* (TP309) line is resistant to the antibiotic hygromycin. Only two UNRR ITLs were obtained after repeated experiments. One line was weak and produced no progeny; the other UNRR(*Xa21*) line (line 49) was healthy and yielded fertile seeds for progeny analysis.

In order to obtain more transgenic UNRR(*Xa21*) lines we switched to using the *phosphomannose isomerase* (*PMI*) gene as the selectable marker. Twenty UNRR(*Xa21*) lines were obtained in this experiment; all carried the *Ubi–NRR* gene as confirmed by PCR of *Ubi–NRR*. Plants of these 20 ITLs were challenged with Xoo PR6. Figure 6(a) demonstrates that nearly all of the 20 UNRR(*Xa21*) ITLs displayed enhanced susceptibility to Xoo PR6 with lesions ranging in length from 5–18 cm. Leaves from three lines (14, 17 and 19) and the *Xa21* recipient control are shown in Figure 6(b). While the *Xa21* control was highly resistant, showing short lesions (approximately 3 cm), inoculated leaves of lines 14, 17 and 19 developed typical water-soaked, long lesions (approximately 15 cm), similar to that observed for TP309.

To confirm that the observed phenotype in UNRR(*Xa21*) is due to the *Ubi–NRR* transgene, the T₁ progeny of UNRR(*Xa21*) line 49 were analyzed by PCR and Xoo inoculation for co-segregation (Figure 6c). The progeny of line 49 segregated for the presence of the *Ubi–NRR* gene, as shown by the PCR results (hybridized with the NRR probe) below the bar graph. The progeny also segregated in their response to Xoo PR6. Those progeny that contain the *Ubi–NRR* gene (in green), labeled UNRR(*Xa21*), show susceptibility whereas the null segregants (in yellow), labeled UNRR–(*Xa21*), retain the *Xa21* resistance. These data confirm that the susceptible phenotype is caused by the *Ubi–NRR* transgene.

To further characterize the effects of UNRR on *Xa21* resistance, Xoo growth curves were carried out for the UNRR(*Xa21*) and UNRR–(*Xa21*) progeny and the *Xa21* and TP309 controls after inoculation with Xoo PR6. Lesion lengths and Xoo populations were measured at 0, 4, 8, 12 and 16 days post-inoculation. Figure 7(a,b) shows the growth curves and results of lesion length measurements. At day 4 no difference was seen between rice lines when Xoo populations had grown to $2 \times 10^7$ colony-forming units per leaf (cfu/leaf). Lesions were also not visible at day 4 (data not shown). From day 4 to day 16, Xoo populations in the *Xa21* control and the UNRR–(*Xa21*) progeny (labeled UNRR–) leveled off to fewer than $5 \times 10^7$ cfu/leaf. In TP309 and the UNRR(*Xa21*) progeny (labeled UNRR), Xoo populations grew to $10^8$–$10^9$ cfu/leaf with leaf lesions appearing at day 6 and spreading quickly until day 16 (see Figure 7b). Xoo growth in UNRR was similar to that in TP309 and more than 10 times higher than that in UNRR–. These results confirmed the effects of UNRR on *Xa21* resistance.

Both NRR mRNA and protein levels are elevated in UNRR lines

To confirm that the enhanced susceptibility phenotype was due to over-expression of the *NRR* gene (NRRox), we first monitored the mRNA levels of *NRR*. The RNA blot hybridization results in Figure 8(a) show that UNRR(*Xa21*) lines 14, 17, 19 and 49 all accumulated high levels of *NRR* mRNA compared with the control, which only expressed low levels...
of NRR mRNA. RNA blot analysis with UNRR lines in TP309 and LG background gave similar results (data not shown).

NRR protein levels of the progeny of UNRR(Xa21) and UNRR(LG) were determined by protein blot analysis using an antiserum generated against the NRR protein. Figure 8(b) (upper panel) shows that NRR protein levels were very low in the Xa21 and UNRR– null segregants (lines 1 and 2), but high in UNRR progeny (lines 3, 4 and 5). Similarly (lower panel), NRR protein levels were low in the UNRR– segregants (lines 3 and 4) of lines 64 and 67 in LG (LG-64 and LG-67) while the UNRR progeny accumulated high levels of NRR protein. These results support the conclusion that the observed phenotype is the consequence of elevated levels of the NRR protein.

The activation of defense-related genes is suppressed in NRRox lines

Because elevated levels of NRR protein negatively regulate resistance to Xoo, we reasoned that NRRox may affect disease resistance by blocking the activation of defense-related genes. We tested this hypothesis by comparing expression of defense-related genes in NRRox lines and the LG control in RNA blot hybridizations.
Total RNA samples were extracted from two groups of 10-week-old rice plants. The first group contains untreated NRRox(LG) lines 64 (NRR64) and 67 (NRR67) as well as the LG control. The second group is same as the first except for inoculation with Xoo PR6. Leaf tissues were collected 4 days after inoculation. Total RNA on blots was hybridized sequentially with probes to PR-1 (Qi and Yang, 1999), peroxidase (POX22.3; Chittoor et al., 1997), NRR and 25S rRNA, or sequentially with probes to PBZ1 (PR-10) (Qi and Yang, 1999), lipoxygenase (LOX2osPil; Peng et al., 1994), NRR and 25S rRNA. Figure 9 shows that, in the LG control, the defense-related genes PR-1, POX and LOX were expressed at very low levels in untreated samples but highly induced after inoculation with Xoo PR6. In contrast, in NRR64 and NRR67, induction of these three genes was obviously suppressed. Induction of the PBZ1 gene was only slightly suppressed in the NRRox lines. Hybridization with the NRR probe confirmed previous results that NRR mRNA is highly elevated in NRR64 and NRR67. It is unclear why NRR mRNA levels in NRR64 and NRR67 are significantly lower in the Xoo-inoculated samples than in the untreated ones. Nevertheless, the results show that NRRox suppresses the activation of the defense-related genes, PR-1, peroxidase and lipoxygenase, and, to a lesser extent, PBZ1.

An NRR:GFP fusion protein is localized to the nucleus

The NRR protein contains a putative nuclear localization sequence KRKR. We tested to see if the NRR protein is transported to the nucleus. We generated a construct to fuse green fluorescent protein (GFP) to NRR. This DNA construct was delivered to onion epidermal cells by biolistic bombardment. As a control, a plasmid expressing the GFP protein alone was delivered into the cells separately. Figure 10(a) shows the green fluorescence (left) and the bright field (right) images of epidermal cells bombarded.

Figure 7. Xoo growth curves and lesion development.
Progeny of UNRR (Xa21) line 49, with (UNRR) or without (UNRR–) the Ubi-NRR transgene, and the Xa21 and TP309 controls were inoculated with Xoo PR6. Leaves were collected at days 0, 4, 8, 12 and 16. Four leaves from each were ground up to measure Xoo populations for growth curves (a) after measuring the lengths of the lesions (b). Each data point represents the average and standard deviation of at least four samples.

Figure 8. mRNA and protein levels of NRR.
(a) NRR RNA blot hybridization. Ten micrograms total RNA each from four UNRR(Xa21) lines (UNRR-14, UNRR-17, UNRR-19 and UNRR-49) and the Xa21 control were loaded in each lane and hybridized with an NRR probe. The blot was subsequently hybridized to a 25S rRNA probe.
(b) Immunoblot of the NRR protein. Protein samples were extracted from Xa21 control, progeny of UNRR(Xa21) line 49, and progeny of UNRR(LG) lines 64 and 67 and probed with an antiserum against the NRR protein. Approximately 260 µg protein for the upper panel and 164 µg protein for the lower panel were loaded in each lane. UNRR and UNRR– depictions are the same as above.

Figure 10(a) shows the green fluorescence (left) and the bright field (right) images of epidermal cells bombarded.
with the NRR:GFP fusion construct. The NRR:GFP fusion protein is solely localized to the nucleus (marked by a red arrowhead). By comparison, Figure 10(b) shows that the GFP protein alone is distributed throughout the cell. The results suggest that NRR is a nuclear protein.

**Discussion**

To study the NPR1-mediated pathway in rice we have isolated cDNA clones encoding NRR based on interaction with NPR1. We subsequently isolated two rice NPR1 homologs, NH1 and NH2, by using NRR as the bait in yeast two-hybrid screens (Chern et al., 2005). In the current study, we have identified an NPR1-interacting domain (NI25) composed of 25 amino acids (from 28 to 52). This domain contains limited sequence similarity to both tobacco G8-1 and Arabidopsis NIMIN2, which also interacts with NPR1. The two amino acids (F40 and L44) in NI25 essential for the interaction are conserved among NRR, NIMIN2 and G8-1. NRR and NIMIN2 (Weigel et al., 2001) were both shown to be nuclear proteins; G8-1 is likely to be a nuclear protein since it also possesses a nuclear localization signal.

Surprisingly, neither NI25 nor NRR52 is enough for strong interaction with rice NH1; instead, another region consisting of 24 amino acids (from 53 to 76) is required. These results suggest that although NH1 is similar to NPR1 overall, it has some features not shared by Arabidopsis NPR1. Rice NRR and NH1 may have co-evolved so that the major domain for interaction has shifted from the NPR1-interacting domain to a nearby region. Interestingly, despite millions of years of divergence, NRR still retains the ability to interact with NPR1.

Reduced accumulation of NRR mRNA by RNA interference led to no detectable changes in defense response to Xoo. Similarly, Weigel et al. (2005) reported that silencing or knockout of the NIMIN1 gene, whose product interacts with NPR1, in Arabidopsis had no effect on disease resistance to Pseudomonas syringae pv. maculicola. These lines displayed enhanced PR-1 gene expression after treatment with SA. Weigel et al. (2005) suggested that interaction of NIMIN1 with NPR1 modulates a subset of PR gene expression in Arabidopsis. Our results (not shown) suggested that PR-10 and POX22.3 gene expression were not affected in NRRsi lines in rice. However, we do not exclude the possibility that silencing of NRR in rice may lead to activation of expression of other PR genes. NRR may be one of a group of proteins interacting with NH1 and NH1-like proteins in rice to regulate defense responses. We are currently characterizing other NH1-interacting proteins to determine if any play a redundant role to NRR.
Because loss-of-function approaches do not always yield a phenotype, gain-of-function approaches have been utilized in various studies. For example, although no knockout phenotypes were observed for AtWRKY18, its role in disease resistance was demonstrated by constitutive over-expression of AtWRKY18 using the CaMV 35S promoter. These experiments led to phenotypes ranging from stunted growth to developmentally regulated activation of defense responses (Chen and Chen, 2002). Endogenous NRR mRNA and protein levels are normally low and nearly undetectable in wild-type plants (Figures 8 and 9). When constitutively over-expressed, NRR causes an enhanced susceptibility phenotype with an increase in Xoo growth. NRR affects both basal resistance and age-related resistance, which has been observed in many plants including Arabidopsis (Kus et al., 2002) and rice (Koch and Mew, 1991) but is poorly understood. The NRR-regulated pathway appears, at least partly, to be responsible for age-related resistance. Thus, the NRR gene may serve as a useful molecular marker in studies of age-related resistance in rice.

NRR also compromises Xoo resistance conferred by the Xa21 resistance gene. Given that NRR affects age-related resistance to Xoo, it is not surprising that NRR also compromises Xa21 resistance because Xa21 confers resistance at the adult stage. Adult rice plants, but not seedlings or young plants, show full resistance to Xoo even though Xa21 mRNA is constitutively expressed (Century et al., 1999). It is hypothesized that some factor(s) only present in adult plants is needed for the Xa21 resistance. NRR may directly or indirectly regulate this factor or affect its presence.

Weigel et al. (2005) recently reported that constitutively expressing high amounts of NIMIN1 led to reduced SA-mediated PR gene induction and a compromised SAR, mimicking npr1 phenotypes. Resistance mediated by RPS2, a member of the large family of the nucleotide binding site–leucine-rich repeat (NBS–LRR) proteins, was also affected in these transgenic Arabidopsis plants. These phenotypes were dependent on the ability of NIMIN1 to interact with NPR1. The phenotypes that we observed in transgenic rice over-expressing NRR were very similar to those reported in Arabidopsis. It is remarkable that two proteins from rice and Arabidopsis, respectively, with little resemblance can lead to such high similarity in affecting resistance mediated by both RLK (rice XA21) and NBS–LRR (Arabidopsis RPS2) type resistance proteins. These observations further support that rice and Arabidopsis share a conserved mechanism of regulating NPR1/NH1 function.

NRR evidently interacts with the C-termini of NH1 and NH2 (Chern et al., 2005). The C-termini of NPR1, NH1 and NH2 contain some of the most conserved regions in these proteins (Chern et al., 2005). Given that NRR, NIMIN2 and G8-1 share an NPR1-interacting domain, interaction with this highly conserved region in NPR1, NH1 and NH2 may have a crucial, fundamental role. NRR may cause the enhanced susceptibility phenotype by inhibiting NH1 and NH2 simultaneously. We cannot rule out the possibility that at unusually high levels NRR suppresses defense responses by interfering with functions of as-yet unidentified proteins (other than NPR1-like proteins). In this case, these unidentified proteins would have to be involved in defense response signaling. Identifying these proteins may reveal other components in defense response pathways.

The EAR motif (LDLN1/2xP) of ERF transcription factors required for active repression of transcription is embedded near the C-terminal ends in the repression domains of these transcription factors (Ohta et al., 2001). The putative EAR motifs (LDLNxxP) in NRR, NIMIN2 and G8-1 are characteristically located near their C-terminal ends. The fact that NRR, NIMIN2 and G8-1 all contain a nuclear localization signal and a putative EAR domain indicates they may function as transcription repressors, possibly to regulate the functions of NPR1 and related proteins. This notion is certainly consistent with the existing results. NIMIN1 (containing the sequence LDLN1) shares only the core of the EAR motif. Whether these sequences function as a repression motif remains to be determined.

What is the selective advantage of suppressing defense responses? Programmed cell death or hypersensitive response normally accompanies defense responses. Mutants and transgenics with misregulated, untimely or over-active defense responses tend to lead to a lesion mimic phenotype (reviewed by Lorrain et al., 2003; Yin et al., 2000). For example, elevated levels of expression of NH1 in rice lead to spontaneous activation of defense genes and lesion mimic phenotypes (Chern et al., 2005). Rice contains unusually high basal levels of SA (Silverman et al., 1995). Keeping defense responses in check in rice may be especially challenging. The presence of NRR and related proteins may serve the purpose of keeping defense responses in check, which is essential for normal plant development.

**Experimental procedures**

**Plant materials and growth conditions**

Rice (*Oryza sativa* L.) plants were maintained in the greenhouse. The growth chamber was set on a 14 h daytime period, a 28/26°C temperature cycle, and at 90% humidity.

**Xoo inoculation and determination of bacterial populations**

For Xoo inoculation, rice plants were grown in the greenhouse normally until they were 6 weeks old (unless stated otherwise) and transferred to the growth chamber. The Xoo strain PX099 (Philippine race 6, PR6) was used to inoculate rice by the scissors-dip
method (Kauffman et al., 1973). Only the top two to three expanded leaves of each tiller were inoculated. For Xoo colony counts from inoculated leaves, 20 cm of leaf tissue from the top, including lesions and tissue showing no lesions, was ground up and suspended in 10 ml H₂O to harvest bacteria. The extract was diluted accordingly and plated out on peptone sucrose agar (PSA) plates containing 15 mg l⁻¹ cefalexin.

Rice transformation

Rice transformation was as described before (Chern et al., 2001). Agrobacterium EHA105 was used to infect rice callus for transformation. Transformation of the rice cultivars TP309 and LG used hygromycin selection. For transformation of TP309 carrying Xa21, which was resistant to hygromycin, we used the Bar (Toki et al., 1992) or the PMI gene for selection as described before (Lucca et al., 2001).

Plasmid construction for NRR over-expression and silencing in rice

A 500 nt cDNA fragment encoding full-length NRR protein was amplified from the original yeast two-hybrid pAD–GAL4 clone using primers mn45-5 (AAGGATCGCA GATCTTCCGAGTGA GAG; and mn45-4 (AGGATCCACT AGTCTCGAGT TGTAATCCGT GAGCA). The PCR product was cloned into pBluescript II SK– using BamHI and SpeI enzymes and the insert confirmed by sequencing.

For over-expression in rice, the NRR cDNA insert was excised by BamHI and SpeI and cloned into the Ubi-C1300 vector, which was created in the same way as Ubi-C1301 (Chern et al., 2001), pre-cut by the same enzymes to create plasmid Ubi-NRR/C1300 (hygromycin selectable). The BamHI/SpeI-digested NRR cDNA was also cloned into the same sites in Ubi-C3000, which was generated in the same way as Ubi-C1301 and carries the same multiple cloning sites, to create Ubi-NRR/C3300 (Bar selectable). To use mannose selection, we created the new plasmid vector C4300 by replacing the gene for hygromycin resistance with the PMI gene (amplified from Escherichia coli) using the Xhol enzyme. We generated the Ubi-C4300 vector by cloning the Ubi promoter-Nos 3’ cassette into C4300 as before. The same NRR cDNA fragment was cloned into it to create Ubi-NRR/C4300 (mannose selectable).

For NRR silencing, a 530 nt NRR fragment (excluding the 220 nt 3’ end) was excised from the original clone in pAD–GAL4 with EcoRI and Nhel and ligated with a 1 kb EcoRI-digested GUS fragment into pBluescript II SK–, pre-cut with XbaI. This cloning resulted in a 2.06 kb insert containing NRR as the ends in reverse orientation, with the open reading frame going outward and GUS in the center as a spacer. After cutting with NotI and NruI to check the orientation, a clone was selected in which the N-terminus of GUS was close to the Saci cloning site. The insert was excised with Saci and SpeI and cloned into the Ubi-C3000 vector pre-cut with the same enzymes.

Plasmid construction for yeast two-hybrid screening

The LexA:NPR1 fusion construct has been described (Chern et al., 2001). To create a LexA:NH1 fusion construct, a 2 kb, full-length NH1 cDNA was excised with EcoRI and Xhol and cloned into plasmid pLex, pre-digested by EcoRI and SacI enzymes. Full-length wild-type NRR cDNA was cloned into the pB42AD vector (Clonetech) via EcoRI and Xhol sites. The N125 fragment was amplified with primers mn45-10 (TTACTAGTCT CGAAGATGCTT GGTGCGGCTG CGCAT) cloned into SK– and sequenced. The N125 insert was then subcloned into pB42AD via EcoRI and Xhol sites.

To create the S39K point mutation, two half pieces of NRR were amplified with primers mn45-6 (CCGGCGGAT CCACAGAGCGA) and mn45-4, yielding NRR-C, and primers mn45-5 and mn45-9 (GGCGCGGAG GATGGCGTAG AACTCCGCA CCGCCGGT), yielding the S39K–, separately; the PCR products were purified and annealed together to generate full-length E39K mutant. The mutant was cloned into SK–, sequenced and subcloned into the pB42AD vector. The N-half of the F40G mutant was amplified with primers mn45-5 and mn45-8 (GGCGCGGAG GATGGCGTAG AACTCCGCA CCGCCGGT) and that of L44G mutant by primers mn45-5 and mn45-7 (GGCGCGGAG GATGGCGTAG AACTCCGCA CCGCCGGGc GATGGCGTAG AACTCCGCA CCGCCGGT) into the pB42AD vector, pre-digested with EcoRI and Xhol.

The pGFP-PL plasmid contains GFP under the control of the CaMV 35S promoter and a Nos terminator for gene expression in plants. NRR was amplified by PCR with primers PN1-1 (GGTCTAGACT CGAAGCAGTC GGGCGCAGGG CGGTCCGGG) and PN1-5 (GGTCTAGACT CGAAGCAGTC GGGCGCAGGG CGGTCCGGG) to create in-frame cloning into plasmid pGFP-PL. PCR products were subcloned into the pCR-Blunt-TOPO vector (Invitrogen, San Diego, CA, USA) and the insert was verified by sequencing. The NRR fragment was released by cutting with the BamHI and Xhol and cloned into the pGFP-PL vector pre-digested with the same enzymes to create plasmid pHF12 for bombardment assays.

Generation of an antiserum against NRR

The full-length NRR cDNA was cloned in-frame into the pET15b protein expression vector. Full-length NRR (131 amino acids) with a 6 x His tag was expressed in E. coli and purified with Ni-NTA agarose resins. The purified His-tagged NRR was used to inject rabbits for raising antiserum. The antiserum was tested against E. coli protein extracts with NRR and without NRR to confirm specificity. Specificity of the antiserum was further verified by probing yeast protein extracts with NRR and without NRR protein (see Figure S2). For protein blotting analysis, the antiserum was diluted at 1:2000. A protein extracts with NRR and without NRR protein (see Figure S2).

PCR, DNA and RNA blot hybridization

Extraction of rice genomic DNA was done according to a protocol described previously (Dellaporta et al., 1984). PCR of the Ubi–NRR transgene was carried out with the maize ubiquitin promoter-specific primer Ubi-1 (TGATATACCT GGAATGATGC A) and NRR-specific primer mn45-14 (GCGTCGCGCA TGCGGCGGAG GATGGCGTAG AACTCCGCA CCGCCGGT) into the pB42AD vector. DNA and RNA blotting and hybridization were performed as described before (Chern et al., 2001). The rice PR-1 (Chern et al., 2005) and PBZ1/PR-10 (Fitzgerald et al., 2004) probes were as described before.
Immuno-coprecipitation

For immuno-coprecipitation, protein was extracted from yeast cells using the Cellying Y buffer (Sigma, St. Louis, MO, USA) supplemented with 1 mM EDTA and protease inhibitor cocktails (Roche, Mannheim, Germany). Forty milliliters of yeast cells were cultured to log phase in a synthetic medium containing galactose to induce protein expression. Yeast cells were spun down and resuspended in 120 µl of buffer. Yeast cells were vigorously vortexed with 200 mg of glass beads. Cell debris and glass beads were spun down. Ninety microliters of the supernatant (approximately 900 µg of protein) was mixed with 0.3 µl of an anti-HA monoclonal antibody (Covance, Cumberland, VA, USA) on ice for 60 min. Ninety microliters of protein G-Dynabeads (Dynal, Oslo, Norway), resuspended in 0.1 M Na acetate (pH 5.0), were added to the protein sample and the reaction incubated on ice for another 60 min. The precipitated protein was washed three times with 0.1 M Na acetate (pH 5.0) according to the manufacturer (Dynal). Protein was eluted in 40 µl of 1x SDS loading buffer by heating up at near boiling for 10 min. The co-precipitated LexA:NHL protein was detected with an anti-LexA monoclonal antibody (Clonetech) after being run on an 8% SDS PAGE and blotted to a nitrocellulose membrane.

Biolistic bombardment of onion epidermal cells and fluorescent microscopy

Onion epidermal tissues were prepared by slicing white onions into 1 in (2.54 cm) square sections. The inner epidermal layer was peeled off and placed inside-up on MS-agar plates (1x Murashige and Skoog medium from Sigma, supplemented with 30 g litre⁻¹ sucrose, 2% agar, pH 5.7). Particle bombardment was done using the protocol of Varagona et al. (1992) and the helium Biolistic™ particle delivery system, PDS-1000/He (Bio-Rad, Hercules, CA, USA), according to the manufacturer’s instructions. In brief, plasmid DNA was precipitated onto 1.6 μm gold particles by mixing CaCl₂ and spermidine. The particles were washed with 70% ethanol, sonicated and resuspended in 100% ethanol. Onion sections were bombarded at 1350 psi (9308 kPa) and 28 in Hg. Onion sections were viewed under a fluorescent microscope (Leica, Wetzlar, Germany) with a bright field or a GFP filter (B2E). Images were obtained using ImagePro software.

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Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Inoculation results of NRRsi progeny from lines #41 (a) and #72 (b). Plants were inoculated at 6 weeks old with Xoo PR6. Lesion lengths were measured 2 weeks later. Each bar represents the average and standard deviation of at least three leaves.

**Figure S2.** Immunodetection by an anti-NRR antibody. Protein extracted from yeast expressing B42AD (vector), B42AD:NRR (NRR), B42AD:FK (EK, NRR mutant), B42AD:FG (FG), B42AD:LG (LG), or B42AD:N125 was probed with either an anti-NRR antibody (a) or with an anti-HA tag antibody (b).

References


