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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 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 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
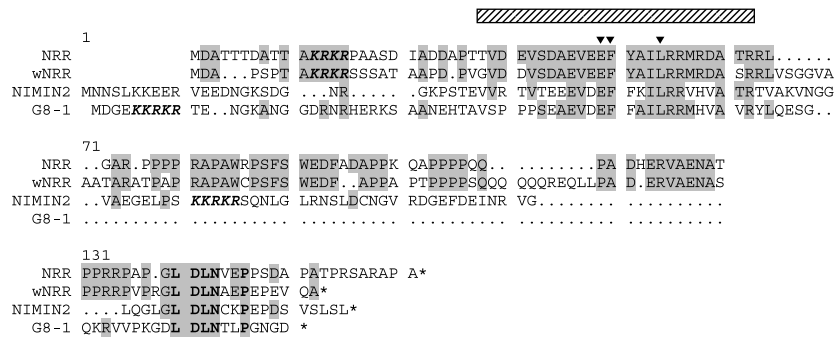
sequence, relatively little is known about the pathways and mechanisms leading to disease resistance, including the *NH1*-mediated pathway. Rice is different from tobacco and Arabidopsis in that it has very high basal levels of SA, and no changes in SA levels were detected after interactions with avirulent 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: 5795981), 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

```
                1
        NRR                MDATTTDATT AKRKRPAASD IADDAPTTVD EVSDAEVEEF YAILRRMRDA TRRL......
        wNRR               MDA...PSPT AKRKRSSSAT AAPD.PVGVD DVSDAEVEEF YAILRRMRDA SRRLVSGGVA
        NIMIN2 MNNSLKKEER VEEDNGKSDG ...NR..... .GKPSTEVVR TVTEEEVDEF FKILRRVHVA TRTVAKVNGG
        G8-1    MDGEKKRKR TE..NGKANG GDRNRHERKS AANEHTAVSP PPSFAEVDEF FAILRRMHVA VRYLQESG..

                71
        NRR    ..GAR.PPPP RAPAWRPSFS WEDFADAPPK QAPPPPQQ.. ........PA DHERVAENAT
        wNRR   AATARATPAP RAPAWCPSFS WEDF..APPA PTPPPPSQQQ QQQREQLLPA D.ERVAENAS
        NIMIN2 ..VAEGELPS KKRKRSQNLG LRNSLDCNGV RDGEFDEINR VG........ ..........
        G8-1   .......... .......... .......... .......... .......... ..........

                131
        NRR    PPRRPAP.GL DLNVEPPSDA PATPRSARAP A*
        wNRR   PPRRPVPRGL DLNAEPEPEV QA*
        NIMIN2 ....LQGLGL DLNCKPEPDS VSLSL*
        G8-1   QKRVVPKGDL DLNTLPGNGD *
```

Figure 1. Protein sequence alignment of NRR, wNRR, NIMIN2 and G8-1.
Protein sequences of rice NRR, wheat NRR (wNRR) homolog, Arabidopsis NIMIN2 and tobacco G8-1 were aligned using the pile-up program of the Wisconsin GCG package. Alignment gaps are filled with dots. Amino acids identical to NRR are shaded. Putative nuclear localization signal sequences are in bold italic face. Putative ERF-associated amphiphilic repression (EAR) motifs are in bold; gaps are introduced manually to align the putative EAR motifs. The hatched bar delineates the NPR1-interacting domain, NI25. Locations of EK, FG and LG mutants are marked by arrowheads.

(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

(a)

NPR1


(b)

NH1   **NH1**

(c)
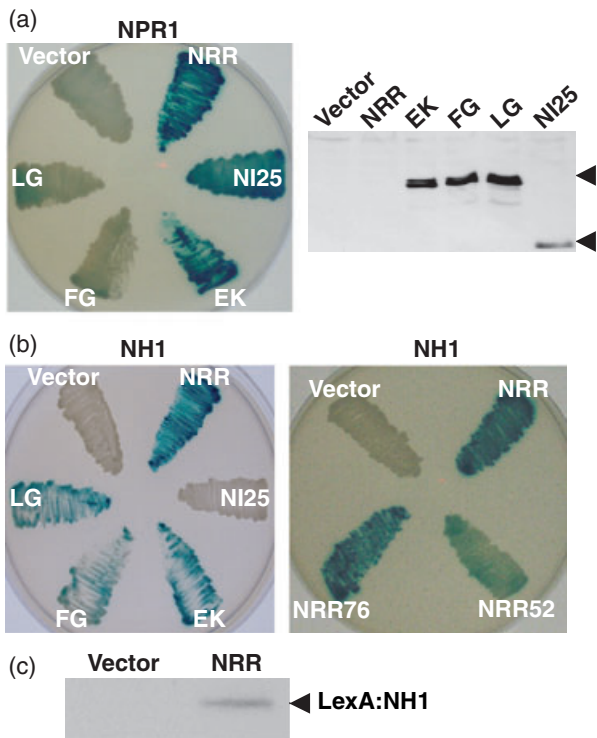
Figure 2. Protein interaction of NRR with NPR1 and NH1.
(a) Left panel: X-gal on-plate detection for interaction of NPR1 with full-length NRR and variants. Right panel: immunodetection of NRR variant proteins in yeast. The NPR1 bait was expressed as a LexA:NPR1 fusion protein; NRR and variants were expressed as a fusion protein to the B42 activation domain (B42AD). Yeast cells harbored NPR1 and either the full-length NRR, a 25-amino-acid NRR peptide (NI25), point mutation E39K (EK), F40G (FG), L44G (LG) or an empty vector as control. The picture was taken 2 days after cells were patched on the X-gal plate. Protein samples were extracted from these cells individually and probed with antibodies against the HA-tag introduced to the fusion protein on the pB42 vector.
(b) X-gal on-plate assay for interaction of NH1 with NRR and variants. NI25, EK, FG and LG are same as above. NRR52 contains the first 52 amino acids and NRR76 the first 76 amino acids of NRR.
(c) Immuno-coprecipitation of yeast-expressed NH1 and NRR proteins. Protein extracted from yeast cells, expressing either LexA:NH1 and B42AD:NRR (labeled NRR) or LexA:NH1 and B42AD (labeled vector), was mixed with an antihemagglutinin (anti-HA) (HA-tag on B42AD) monoclonal antibody and pulled down by protein G-magnetic beads. The precipitated protein was run in a SDS gel, blotted to a membrane and probed with an anti-LexA antibody (Clontech).

in rice. 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 T_1

(a)

(b)
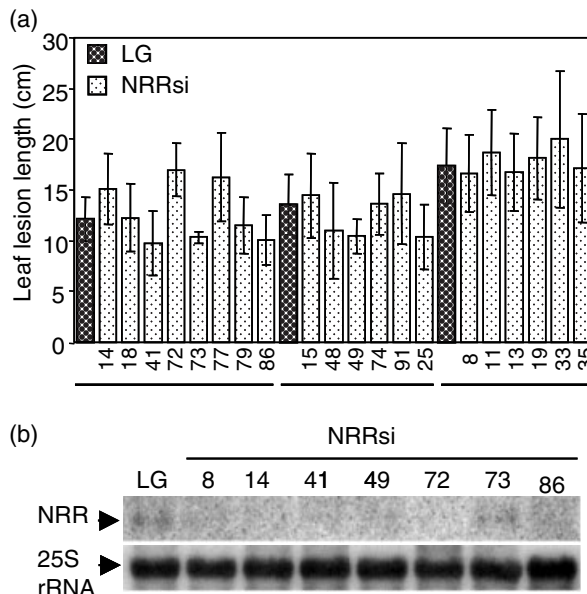
Figure 3. *Xoo* inoculation results and RNA blot analysis of NRRsi lines.
(a) Length of leaf lesions in inoculated transgenic plants carrying an RNA interference construct targeting NRR (labeled NRRsi). Leaves were inoculated with *Xoo* 6 weeks after regeneration; lesion lengths were measured 2 weeks afterwards. Each bar represents the average and standard deviation of at least three leaves. The number under each bar depicts an independent transgenic line. The transgenic lines were inoculated in three batches (grouped by lines under the numbers), each with the LG control.
(b) RNA blot analysis. RNA samples extracted from 5-week-old plants of seven putative NRRsi lines and LG were hybridized with *NRR* and *25S rRNA* probes sequentially. NRRsi line numbers are indicated.

progeny of lines 41 (slightly more resistant) and 72 (slightly more susceptible) were inoculated with *Xoo* PR6 to determine if the phenotypes in T_0 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
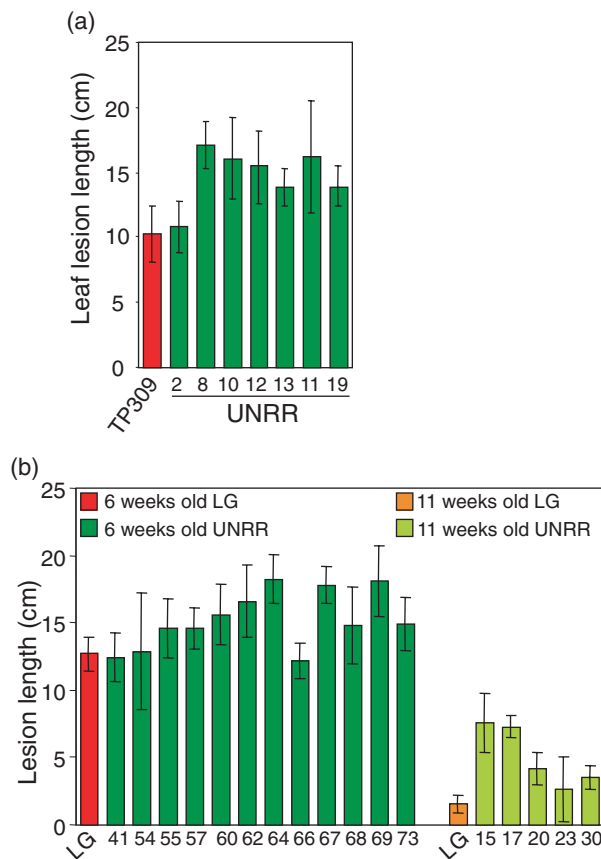
Figure 4. Xoo inoculation results of *Ubi–NRR* (UNRR) transgenic plants.
(a) length of lesions in Xoo-inoculated UNRR (TP309) T_0 plants and TP309 controls. Leaves were inoculated when the plants were 6 weeks old; lesion lengths were measured 2 weeks afterwards. Each bar represents the average and standard deviation of at least five leaves. (b) Lesion length of inoculated UNRR (LG) T_0 plants and LG control. Plants were inoculated at 6 or 11 weeks of age (as indicated); lesion lengths were measured 2 weeks afterwards. Each bar represents the average and standard deviation of at least five leaves.

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
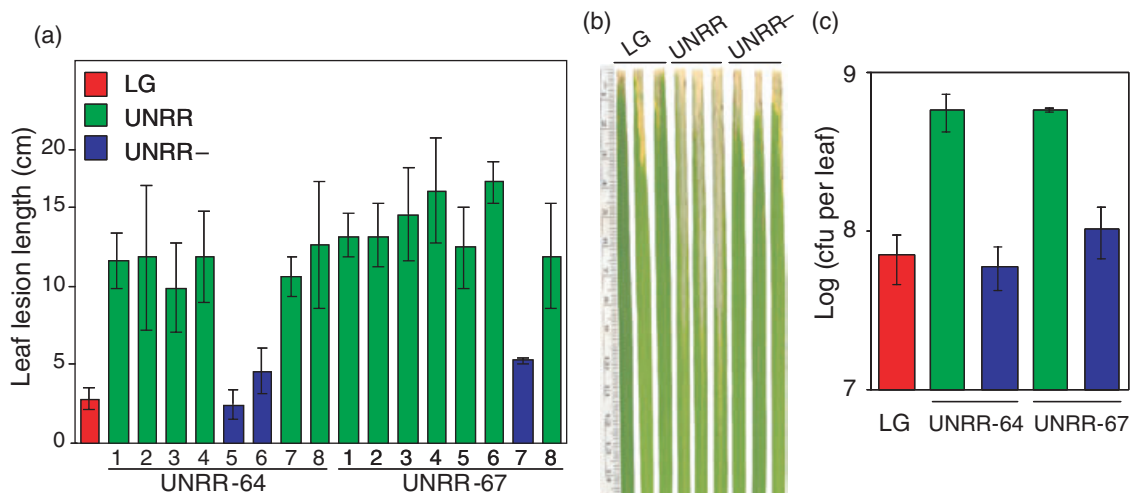
Figure 5. Progeny segregation analysis of UNRR lines 64 (UNRR-64) and 67 (UNRR-67) in an LG rice cultivar.
(a) Lengths of lesions in segregation progeny inoculated with Xoo PR6. Progeny retaining the *Ubi–NRR* transgene are labeled UNRR; null segregants that have lost the transgene are labeled UNRR–. Each bar represents the average and standard deviation of at least four leaves.
(b) Picture of leaf lesions 2 weeks after inoculation.
(c) Xoo populations. Two weeks after inoculation, Xoo populations were determined for each individual leaf. Each bar represents the average and standard deviation of four samples.

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 2×10^7 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 5×10^7 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

(a)

(b)

(c)
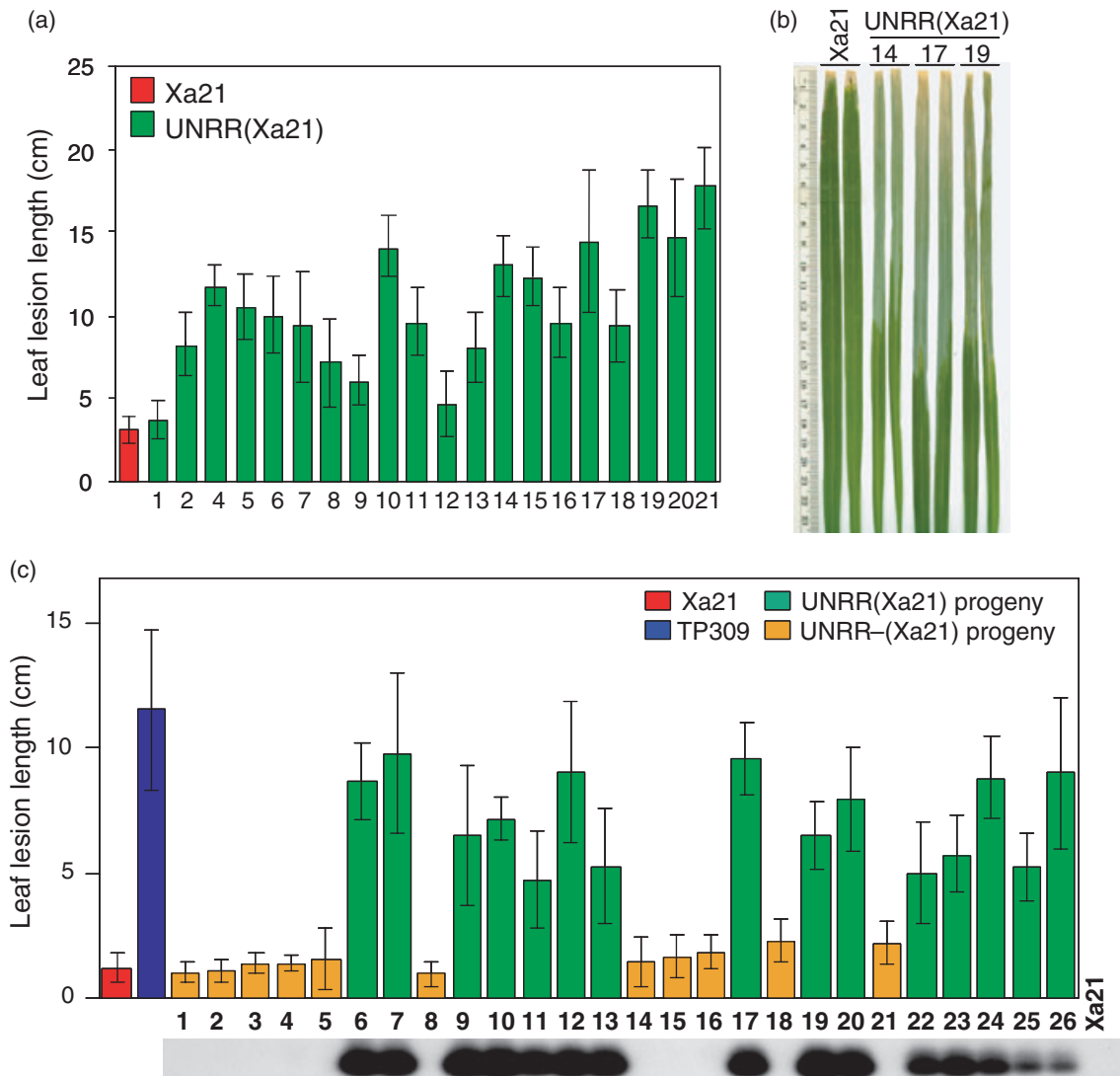
Figure 6. Xoo inoculation results of UNRR(*Xa21*) and progeny segregation analysis.
(a) Twenty independent T_0 lines carrying the *Ubi–NRR* transgene in the *Xa21* background, UNRR(*Xa21*), together with the *Xa21* control were inoculated with Xoo PR6 at 6 weeks of age. The lengths of the lesions were measured 2 weeks afterwards. Each bar represents the average and standard deviation of at least five leaves.
(b) Water-soaked disease lesions on two leaves each of three UNRR(*Xa21*) lines (lines 14, 17 and 19) and the *Xa21* control are shown 2 weeks post-inoculation.
(c) Segregation analysis of progeny of UNRR(*Xa21*) line 49. Plants were inoculated at 6 weeks of age and the lengths of the lesions measured 2 weeks afterwards. Each bar represents the average and standard deviation of at least four leaves. Hybridization of PCR products with an *NRR* probe is shown under the graph for each segregant. Progeny carrying the *Ubi–NRR* transgene are labeled UNRR and null segregants labeled UNRR–.

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.

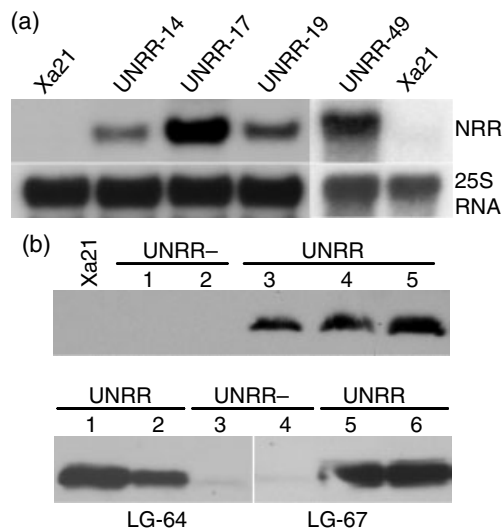*The activation of defense-related genes is suppressed in NRRox lines*

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.
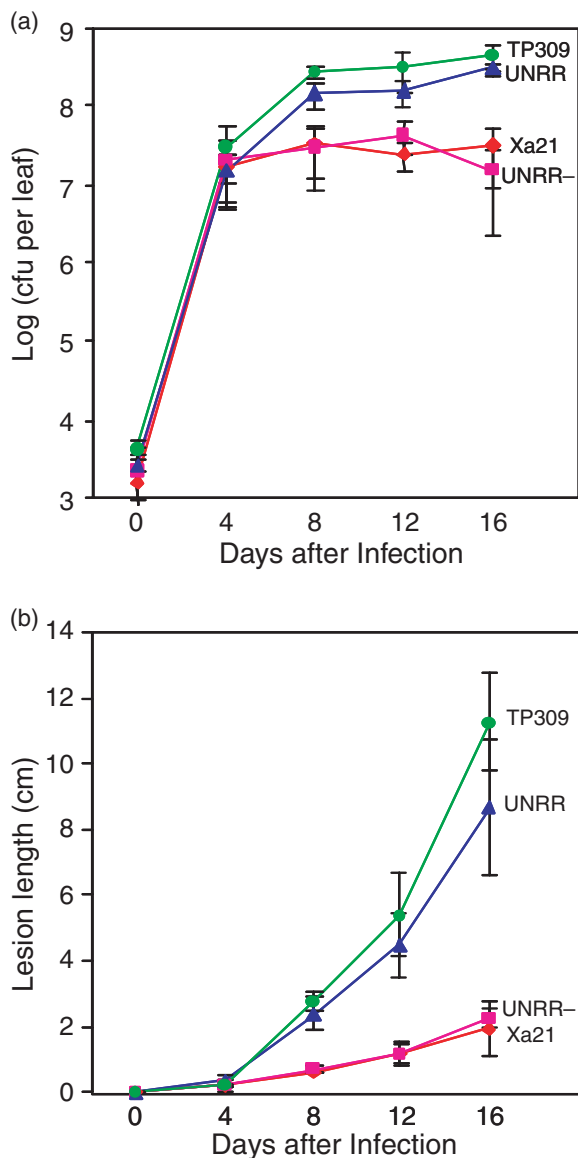
Figure 7. Xoo growth curves and lesion development.
Progeny of UNRR (*Xa21*) line 49, with (UNRR) or without (UNRR–) the *Ubi–NRR* transgene, and the *Xa21* and TP309 controls were inoculated with Xoo PR6. Leaves were collected at days 0, 4, 8, 12 and 16. Four leaves from each were ground up to measure 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 250 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.

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* (*PR-1b* probe; 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
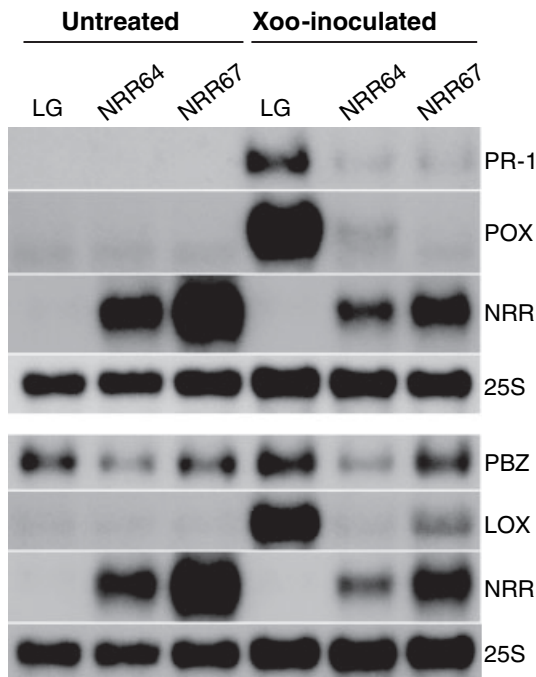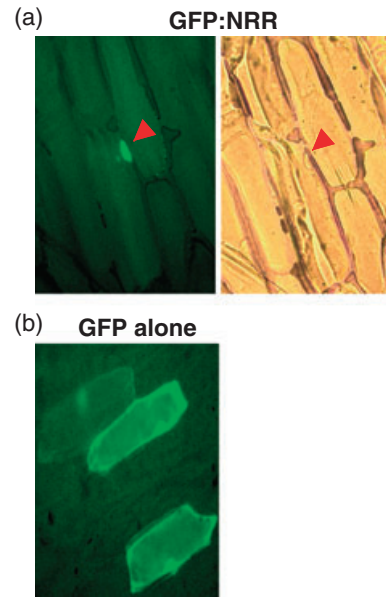
Figure 9. Effects of NRR over-expression on RNA accumulation of defense-related genes.
Total RNA samples were extracted from 10-week-old rice plants of LG control, NRRox(LG) lines 64 and 67 (NRR64 and NRR67), untreated or inoculated with Xoo PR6. For the Xoo-inoculated samples, leaf tissues were collected 4 days after inoculation. Ten micrograms total RNA were loaded in each lane. Total RNA on two separate blots was hybridized sequentially with probes to *PR-1*, peroxidases (*POX*), *NRR* and 25S rRNA, or sequentially to *PBZ1* (*PR-10*), lipoxygenases (*LOX*), *NRR* and 25S rRNA.

Figure 10. Subcellular localization of NRR:GFP fusion protein.
(a) NRR was fused to GFP in the pGFP-PL plasmid. The NRR:GFP plasmid DNA was delivered to onion epidermal cells by biolistic bombardment. Left panel: dark field; right panel: bright field.
(b) As a control, the pGFP-PL plasmid alone was delivered separately.

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 syriage* 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 what Weigel et al. (2005) have 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 (LDLN^L/$_F$xP) 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 LDLNL) 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 PXO99 (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 resuspended in 10 ml H_2O to harvest bacteria. The extract was diluted accordingly and plated out on peptone sucrose agar (PSA) plates containing 15 mg l^{-1} cephalexin.

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 (AAGGATCCAA GAATTCACCA CCACCATGGA CGC) and mn45-4 (AGGATCCACT AGTCTCGAGT TGTAATCCGT GAGCA). The PCR product was cloned into pBlueScript II SK– using *Bam*HI and *Spe*I enzymes and the insert confirmed by sequencing.

For over-expression in rice, the *NRR* cDNA insert was excised by *Bam*HI and *Spe*I 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 *Bam*HI/*Spe*I-digested NRR cDNA was also cloned into the same sites in Ubi-C3300, 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 *Xho*I 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 *Eco*RI and *Nhe*I and ligated with a 1 kb, *Eco*RI-digested GUS fragment into pBlueScript II SK–, pre-cut with *Xba*I. This cloning resulted in a 2.06 kb insert containing NRR at the ends in reverse orientation, with the open reading frame going outward and GUS in the center as a spacer. After cutting with *Not*I and *Nru*I to check the orientation, a clone was selected in which the N-terminus of GUS was close to the *Sac*I cloning site. The insert was excised with *Sac*I and *Spe*I and cloned into the Ubi-C1300 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 *Eco*RI and *Xho*I and cloned into plasmid pNLex, pre-digested by *Eco*RI and *Sal*I enzymes. Full-length wild-type *NRR* cDNA was cloned into the pB42AD vector (Clontech) via *Eco*RI and *Xho*I sites. The NI25 fragment was amplified with primers mn45-11 (AAGGATCCAA GAATTCACCG TCGACGAGGT CT) and

mn45-10 (TTACTAGTCT CGAGCTATCG GGTGGCGTCG CGCAT), cloned into SK– and sequenced. The NI25 insert was then subcloned into pB42AD via *Eco*RI and *Xho*I sites.

To create the E39K point mutation, two half pieces of *NRR* were amplified with primers mn45-6 (CCGCCGCATG CGCGACGCCA) and mn45-4, yielding NRR-C, and primers mn45-5 and mn45-9 (GCGTCGCGCA TGCGGCGGAG GATGGCGTAG AACTtCTCGA CCTCGGCGT), yielding the N-half, 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 (GCGTCGCGCA TGCGGCGGAG GATGGCGTAG ccCTCCTCGA CCTCGGCGT) and that of L44G mutant by primers mn45-5 and mn45-7(GCGTCGCGCA TGCGGCGGcc GATGGCGTAG AACTCCT). The N-halves and F40G and L44G were joined with NRR-C in the same way as described for E39K, to create full-length F40G and L44G. F40G and L44G were subsequently cloned into pB42AD.

The NRR52 DNA fragment was amplified with primers mn45-5 and mn45-19 (TTGAATTCGC TAGCTCGGGT GGCGTCGCGC AT) and NRR76 was amplified with primers mn45-5 and mn45-20 (TTGAATTCGC TAGCGAAGTC CTCCCAGGAG AA); both were cloned into SK– in the *Eco*RI site and the inserts sequenced. Each insert was cut out with *Eco*RI + *Nhe*I enzymes and, together with an *Nhe*I/*Xho*I-digested VP16 DNA fragment, cloned into the pB42AD vector, pre-digested with *Eco*RI and *Xho*I.

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 PNI1-1 (GCTCTAGACT CGAGACCATG GACGCCACCA CC) and PNI1-5 (CATGCCATGG GGATCCTTTG CCGGGGCGCG CGCCGA) 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 *Bam*HI and *Xho*I 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 $6 \times$ 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 antisera. The antisera were tested against *E. coli* protein extracts with NRR and without NRR to confirm specificity. Specificity of the antisera 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 chemiluminescence substrate system was used to detect NRR in protein blots.

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 (TGATATACTT GGATGATGGC A) and *NRR*-specific primer mn45-14 (GAGGATGGCG TAGAACTCCT). 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 Cellytic 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 1× SDS loading buffer by heating up at near boiling for 10 min. The co-precipitated LexA:NH1 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 (1× Murashige and Skoog medium from Sigma, supplemented with 30 g l^{-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 CaCl$_2$ 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.

Acknowledgements

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:EK (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

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. USA*, **95**, 6531–6536.

Century, K.S., Lagman, R.A., Adkisson, M., Morlan, J., Tobias, R., Schwartz, K., Smith, A., Love, J., Ronald, P.C. and Whalen, M.C. (1999) Developmental control of *Xa21*-mediated disease resistance in rice. *Plant J.* **20**, 231–236.

Chen, C. and Chen, Z. (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. *Plant Physiol.* **129**, 706–716.

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.

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

Chittoor, J.M., Leach, J.E. and White, F.F. (1997) Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant Microbe Interact.* **10**, 861–871.

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. USA*, **92**, 6602–6606.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1984) Molecular biology of plants. In *A Laboratory Course Manual* (Russell, M., ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, pp. 36–37.

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 TGA family 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.

Fitzgerald, H.A., Chern, M.-S., Navarre, R. and Ronald, P.C. (2004) Overexpression of (At)NPR1 in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Mol. Plant Microbe Interact.* **17**, 140–151.

Friedrich, L., Lawton, K., Ruess, W. *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.

Gorlach, J., Volrath, S., Knauf-Beiter, G. *et al.* (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell*, **8**, 629–643.

Horvath, D.M., Huang, D.J. and Chua, N.H. (1998) Four classes of salicylate-induced tobacco genes. *Mol. Plant Microbe In.* **11**, 895–905.

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.

Kauffman, H.E., Reddy, A.P.K., Hsieh, S.P.V. and Marca, S.D. (1973) An improved technique for evaluation of resistance of rice varieties to *Xanthomonas oryzae*. *Plant Dis. Rep.* **57**, 537–541.

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

Koch, M.F. and Mew, T.W. (1991) Effect of plant age and leaf maturity on the quantitative resistance of rice cultivars to *Xanthomonas campestris* pv. *oryzae*. *Plant Dis.* **75**, 901–904.

Kus, J.V., Zaton, K., Sarkar, R. and Cameron, R.K. (2002) Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae*. *Plant Cell*, **14**, 479–490.

Lorrain, S., Valileau, F., Balague, C. and Roby, D. (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants. *Trends Plant Sci.* **8**, 263–271.

Lucca, P., Ye, X. and Potykus, I. (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Mol. Breed.* **7**, 43–49.

Morris, S.W., Vernoolij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C.C., Frederiksen, R.A., Bhandhufalck, A., Hulbert, S. and Uknes, S. (1998) Induced resistance responses in maize. *Mol. Plant Microbe Interact.* **11**, 643–658.

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.

Peng, Y.L., Shirano, Y., Ohta, H., Hibino, T., Tanaka, K. and Shibata, D. (1994) A novel lipoxygenase from rice. Primary structure and specific expression upon incompatible infection with rice blast fungus. *J. Biol. Chem.* **269**, 3755–3761.

Qi, M. and Yang, Y. (1999) Differential expression of rice PR-1 and PR-10 genes induced by blast fungus, elicitor, and chemical treatments. *Phytopathology*, **89**, S62.

Rohilla, R., Singh, U.S. and Singh, R.L. (2002) Mode of action of aciebnzolar-S-methyl against sheath blight of rice caused by *Rhizoctonia solani* Kuhn. *Pest Manag. Sci.* **58**, 63–69.

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.

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

Schweizer, P., Schlagenhauf, E., Schaffrath, U. and Dudler, R. (1999) Different patterns of host genes are induced in rice by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical inducer benzothiadiazole (BTH). *Eur. J. Plant Pathol.* **105**, 659–665.

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.

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Metraux, J.-P. and Raskin, I. (1995) Salicylic acid in rice: biosynthesis, conjugation, and possible role. *Plant Physiol.* **108**, 633–639.

Smith, J.A. and Metraux, J.-P. (1991) *Pseudomonas syringae* pathovar *syringae* induces systemic resistance to pyricularia oryzae in rice. *Physiol. Mol. Plant Pathol.* **39**, 451–461.

Song, W.-Y., Wang, G.-L., Chen, L., Kim, H.-S., Holsten, T., Zhai, W.-X., Zhu, L., Fauquet, C. and Ronald, P. (1995) The rice disease resistance gene, *Xa21*, encodes a receptor kinase-like protein. *Science*, **270**, 1804–1806.

Toki, S., Takamatsu, S., Nojiri, C., Ooba, S., Anzai, H., Iwata, M., Christensen, A.H., Quail, P.H. and Uchimya, H. (1992) Expression of a maize *ubiquitin* gene promoter-*Bar* chimeric gene in transgenic rice plants. *Plant Physiol.* **100**, 1503–1507.

Varagona, M.J., Schmidt, R.J. and Raikhel, N.V. (1992) Nuclear localization signals required for nuclear targeting of the maize regulatory protein opaque-2. *Plant Cell*, **4**, 1213–1227.

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.

Yin, Z., Chen, J., Zeng, L., Goh, M., Leung, H., Khush, G. and Wang, G.-L. (2000) Characterizing rice lesion mimic mutants and identifying a mutant with broad-spectrum resistance to rice blast and bacterial blight. *Mol. Plant Microbe Interact.* **13**, 869–876.

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. USA*, **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.