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Evidence for a disease-resistance pathway in rice similar to the *NPR1*-mediated signaling pathway in *Arabidopsis*

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Summary

The *Arabidopsis NPR1/NIM1* gene is a key regulator of systemic acquired resistance (SAR). Over-expression of *NPR1* leads to enhanced resistance in *Arabidopsis*. To investigate the role of *NPR1* in monocots, we over-expressed the *Arabidopsis NPR1* in rice and challenged the transgenic plants with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the rice bacterial blight pathogen. The transgenic plants displayed enhanced resistance to *Xoo*. RNA blot hybridization indicates that enhanced resistance requires expression of *NPR1* mRNA above a threshold level in rice. To identify components mediating the resistance controlled by NPR1, we used NPR1 as bait in a yeast two-hybrid screen. We isolated four cDNA clones encoding rice NPR1 interactors (named rTGA2.1, rTGA2.2, rTGA2.3 and rLG2) belonging to the bZIP family. rTGA2.1, rTGA2.2 and rTGA2.3 share 75, 76 and 78% identity with *Arabidopsis* TGA2, respectively. In contrast, rLG2 shares highest identity (81%) to the maize *liguleless* (*LG2*) gene product, which is involved in establishing the leaf blade–sheath boundary. The interaction of NPR1 with the rice bZIP proteins in yeast was impaired by the npr1-1 and npr1-2 mutations, but not by the nim1-4 mutation. The NPR1–rTGA2.1 interaction was confirmed by an *in vitro* pull-down experiment. In gel mobility shift assays, rTGA2.1 binds to the rice RCH10 promoter and to a *cis*-element required sequence-specifically for salicylic acid responsiveness. This is the first demonstration that the *Arabidopsis NPR1* gene can enhance disease resistance in a monocot plant. These results also suggest that monocot and dicot plants share a conserved signal transduction pathway controlling *NPR1*-mediated resistance.

Keywords: rice, *Xanthomonas oryzae*, SAR, NPR1, bZIP, *liguleless*.

Introduction

Plants are able to resist subsequent infection by pathogens after recovery from an initial inoculation with an avirulent pathogen. In dicots such as *Arabidopsis* and tobacco, salicylic acid (SA) is both required and responsible for inducing this long-lasting systemic acquired resistance (SAR). SAR is characterized by broad-spectrum resistance to viral, bacterial and fungal pathogens, and the inducible expression of pathogenesis-related (*PR*) genes (Ryals *et al.*, 1996). In addition to SA, synthetic chemicals such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are potent inducers of SAR in dicots.

It is known that *NPR1* (also known as *NIM1 and SAI1*) is a key regulator of SA-mediated SAR in *Arabidopsis* (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook *et al.*, 1996; Ryals *et al.*, 1997; Shah *et al.*, 1997). So far, *NPR1* is the only gene identified that affects the SAR pathway downstream of the SA signal in *Arabidopsis*. *Arabidopsis npr1/nim1* mutants are impaired in their ability to induce PR gene expression and mount an SAR response even after treatment with SA or INA. On induction by SA, INA or BTH, *NPR1* expression levels are elevated (Cao *et al.*, 1997; Ryals *et al.*, 1997). *NPR1* also participates in the jasmonate- and ethylene-regulated, SA-independent induced systemic resistance (ISR) (Pieterse *et al.*, 1998). The *Arabidopsis NPR1/NIM1* gene encodes a novel protein with ankyrin repeats (Cao *et al.*, 1997; Ryals *et al.*, 1997). Nuclear local-

ization of NPR1 is essential for its function in inducing *PR* gene expression (Kinkema *et al.*, 2000). Over-expression of *NPR1* in *Arabidopsis* leads to enhanced disease resistance to both bacterial and oomycete pathogens in a dose-dependent manner (Cao *et al.*, 1998). Transgenic *Arabidopsis* over-expressing *NPR1* displays no obvious detrimental effects. Thus *NPR1* represents an ideal target for engineering broad-spectrum disease resistance.

It has been reported that NPR1 interacts differentially with the *Arabidopsis* TGA family members of basic-region leucine zipper (bZIP) transcription factors (Despres *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000). Among the *Arabidopsis* TGA members, NPR1 preferentially interacts with TGA2 (also known as AHBP-1b), TGA3, TGA5 and TGA6 (Zhang *et al.*, 1999; Zhou *et al.*, 2000). The ankyrin repeats of NPR1 are necessary and sufficient for the interaction, although high-affinity interactions also require the N-terminal one-third of NPR1 (Zhang *et al.*, 1999). In addition, several mutant npr1/nim1 proteins, such as npr1-1, npr1-2, npr1–5 and nim1–2, that fail to support SAR, also lose their ability to interact with these TGA proteins in the yeast two-hybrid system (Zhang *et al.*, 1999; Zhou *et al.*, 2000). Despres also reports that the interaction between NPR1 and TGA proteins facilitates binding of the TGA proteins to the SA-responsive *as-1* DNA element of the CaMV 35S promoter and the LS5 and LS7 elements of the *PR1* promoter (Despres *et al.*, 2000).

In tobacco, the nuclear factor SARP that interacts with the *as-1* element is immunologically related to TGA1a (Jupin and Chua, 1996). Furthermore, it is demonstrated that tobacco TGA2.2 is the main component of SARP, and that over-expression of TGA2.2 and a dominant negative TGA2.2 affect SA-inducibility of target genes (Niggeweg *et al.*, 2000). Similarly, *Arabidopsis* TGA2 and TGA3 are shown to bind to an SA-responsive element of the *Arabidopsis PR1* promoter that shares the TGACG core sequence for cognate TGA-binding sites with the *as-1* element (Zhang *et al.*, 1999). These results suggest that the *Arabidopsis* and tobacco TGA proteins directly connect NPR1 with *PR* gene induction in the SA signal transduction pathway.

It remains unclear whether the *NPR1*-mediated, SA-dependent defense pathway exists in monocots. There are contradictory observations for the role of SA in monocots. For example, high levels of endogenous SA are detected in rice even under non-inducing conditions (Silverman *et al.*, 1995). However, in wheat, BTH treatment induces acquired resistance systemically to powdery mildew infection and the expression of novel WCI genes (Gorlach *et al.*, 1996). In rice, BTH is also reported to induce resistance to *Magnaporthe grisea*, and expression of a set of genes (Schweizer *et al.*, 1999). Thus both in monocots and dicots it is known that BTH induces resistance to pathogens and expression of a particular group of genes. As *NPR1* is a key regulator of SAR in *Arabidopsis* and represents a target for engineering disease resistance, we sought to determine whether the *Arabidopsis NPR1*-mediated resistance pathway is conserved in economically important crops cereals.

Rice provides the most amenable system in monocots for molecular genetic studies of disease resistance, due to its small genome size, extensive genetic map, large sequence databases, and relative ease of transformation (Hiei *et al.*, 1994; Komari *et al.*, 1998). To study if the *NPR1*-mediated defense pathway functions in rice, we over-expressed the *Arabidopsis NPR1* in rice and challenged the transgenic plants with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of rice bacterial blight. Here we show that enhanced resistance to *Xoo* can be achieved by over-expressing the *Arabidopsis NPR1*. Furthermore, we have isolated rice cDNA clones encoding proteins interacting with NPR1. These rice NPR1 interactors belong to the bZIP transcription factor family and share similarity with the *Arabidopsis* TGA2 protein. Our results suggest the presence of a rice disease-resistance pathway similar to the *Arabidopsis NPR1*-mediated signal transduction.

Results

Transgenic rice over-expressing NPR1 *is resistant to bacterial blight*

To over-express *NPR1* in rice, we constructed *Ubi-NPR1 and 35S-NPR1* plasmids in which expression of the *Arabidopsis NPR1* cDNA was driven by a maize *ubiquitin* (*Ubi*) promoter (Christensen and Quail, 1996) and the CaMV *35S* promoter, respectively. *Agrobacterium*-mediated transformation of the rice cultivar Taipei (TP) 309 was used to generate transgenic plants carrying these two constructs. After regeneration, 6- to 8-week-old rice plants were challenged with *Xoo* Philippine race 6 (PR6) to test for enhanced resistance. We have tested at least 20 *Ubi-NPR1 and* 29 *35S-NPR1* independent transgenic lines.

Figure 1(a) shows the inoculation results of seven *Ubi-NPR1* (2, 3, 6, 7, 10, 18 and 20) and four *35S-NPR1* (5R, 14R, 17R and 18R) primary (T_0) transgenic lines displaying different levels of enhanced resistance. The data were collected from four independent inoculation experiments. In each inoculation, the transformation recipient TP309 and the IRBB21 cultivar or an *Xa21*-transgenic line (Song *et al.*, 1995) were included for comparison. The mean and standard deviation were calculated from at least six leaves for each transgenic line, and for control plants. Both IRBB21 and *Xa21* control plants contain the *Xa21* gene and are resistant to *Xoo* PR6. TP309 control plants are susceptible to *Xoo* PR6 and developed, on average, a leaf lesion length of 9 cm or longer, whereas IRBB21 and *Xa21* plants yielded lesion lengths of 2 cm or shorter. Many of the *Ubi-NPR1* lines developed lesion lengths much shorter than

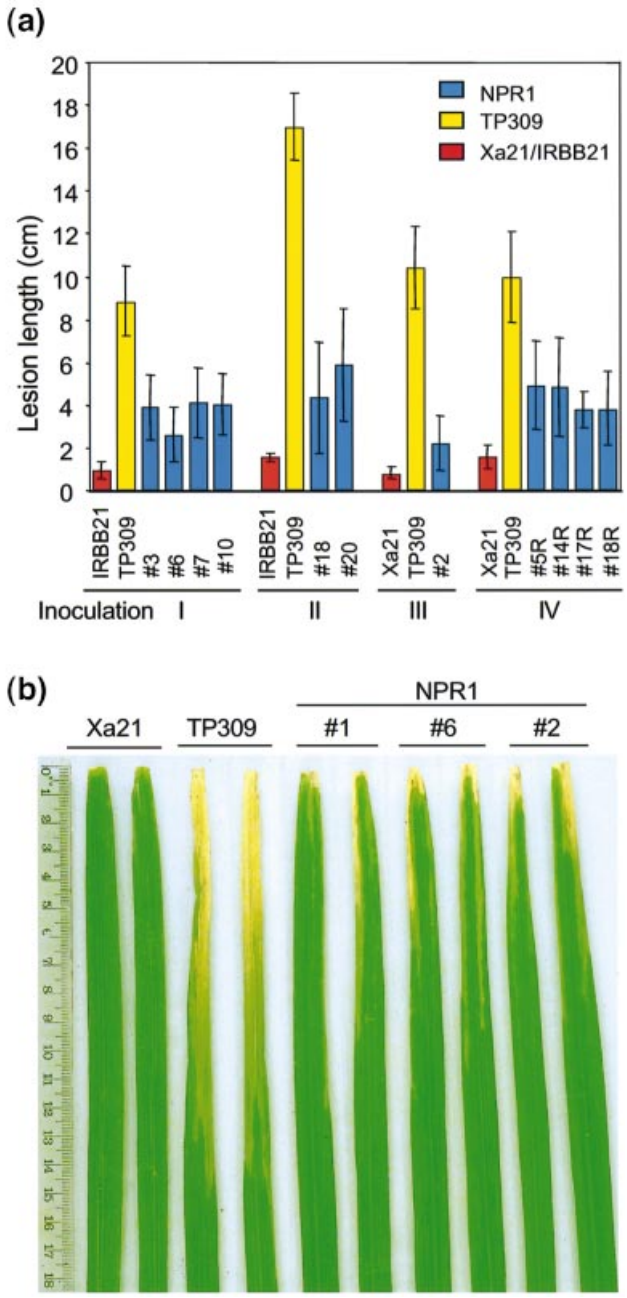

Figure 1. Leaf lesion of *NPR1* transgenic rice challenged with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Philippine race 6.
Leaves of 6- to 8-week-old plants were inoculated with *Xoo* by the scissors-dip method.
(a) Graph of leaf lesion lengths of *NPR1* primary (T_0) transgenic plants. Lesion length was measured 2 weeks after inoculation. Seven T_0 *Ubi-NPR1* (3, 6, 7, 10, 18, 20 and 2) and four *35S-NPR1* (5R, 14R, 17R and 18R) transgenic lines are presented as blue bars; Taipei (TP) 309 (the transformation recipient) as yellow; and IRBB21 and *Xa21* transgenic (in TP309 background) as red. Data were collected from four independent inoculations (I–IV). Each bar represents the average and standard deviation of six or more leaves.
(b) Inoculated leaf samples of *NPR1* plants displaying enhanced resistance. Leaf samples of six resistant T_1 progeny plants derived from three T_0 lines (1, 6 and 2) are shown, along with those of TP309 and transgenic *Xa21* plants. Leaf samples were taken 2 weeks after inoculation.

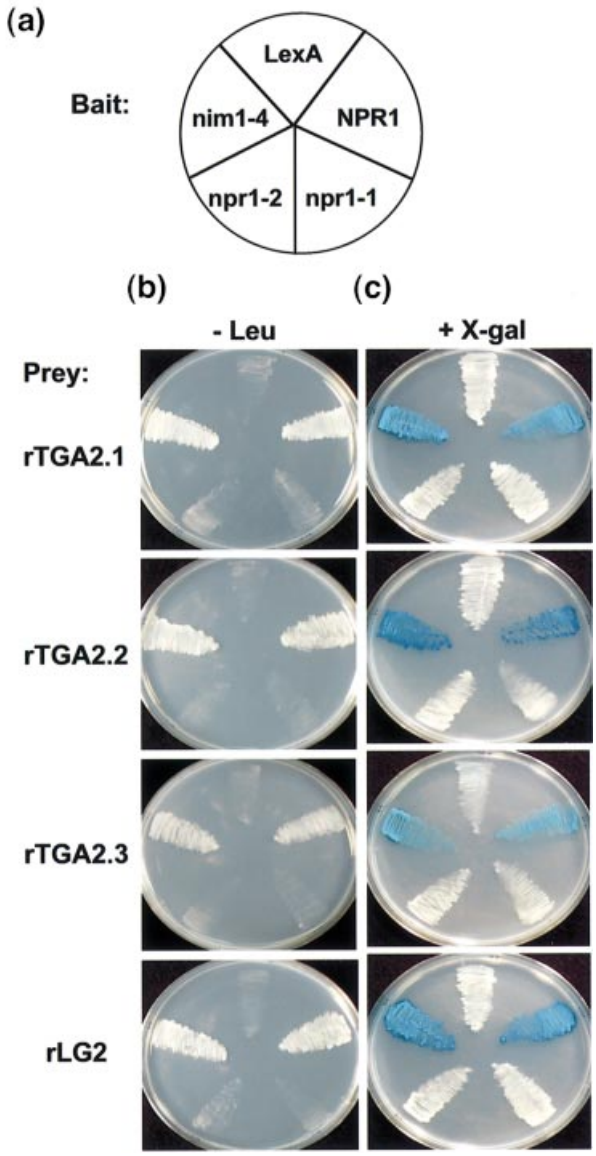

Figure 7. Effects of npr1/nim1 mutations on interaction with rice bZIP proteins.
(a) Schematic diagram of the plate arrangement of yeast cells containing empty bait (LexA), NPR1, npr1-1, npr1-2 and nim1-4 plasmids, respectively.
(b) Leucine (Leu) dropout assay.
(c) β-galactosidase activity assay on plates. X-gal was included in the plates as substrate for the β-galactosidase enzyme. The individual prey construct carried in the yeast cells on each plate is indicated on the left.

that of TP309, indicating that over-expression of *NPR1* in rice can lead to enhanced resistance to bacterial blight. In general, the *Ubi-NPR1* lines displayed a higher level of enhanced resistance than the *35S-NPR1* lines (data not shown). Therefore we conducted further analysis only on the *Ubi-NPR1* lines.

Most of the *NPR1* transgenic lines yielded fertile seed. We tested T_1 plants of several independent lines for

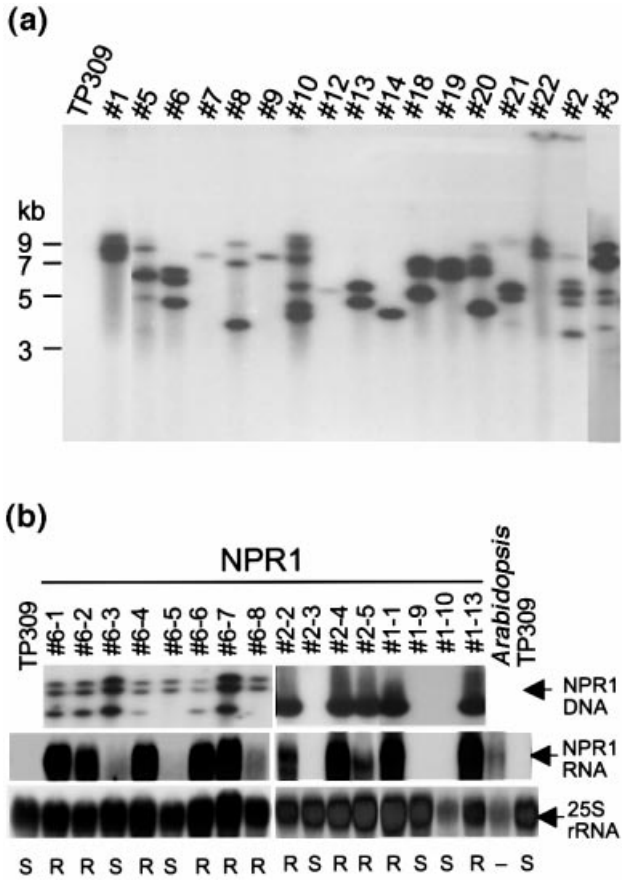

Figure 2. DNA and RNA blot hybridization analyses of *NPR1* transgenic rice.
(a) DNA blot hybridization. Genomic DNA was extracted from *Ubi-NPR1* transgenic plants and TP309 control, digested with *Bam*HI, and hybridized to *NPR1* on blots. The number above each lane indicates the individual transgenic line.
(b) Presence of *NPR1* transgene, RNA expression, and disease resistance rating in T_1 progeny. Genomic DNA blot hybridization was conducted on T_1 progeny of line 6 (6-1 to 6-8) and PCR was carried out on progeny of lines 2 (2-2, 2-3, 2-4 and 2-5), and 1 (1-1, 1-9, 1-10 and 1-13) to assess the presence of the *NPR1* transgene. Total RNA samples were extracted from leaves of these T_1 plants, TP 309, and BTH-treated *Arabidopsis*. The RNA blots were hybridized to *NPR1* and subsequently probed with a rice 25S rDNA for reference. The hybridizing bands corresponding to *NPR1* mRNA and 25S rRNA are indicated. Approximately 10 μg total RNA was loaded in each lane. These T_1 progeny were inoculated with *Xoo* Philippine race 6. Plants with lesion lengths equal to or shorter than 6 cm are rated resistant (R), and those with lesion lengths equal to or longer than 9 cm are rated susceptible (S).

segregation of the enhanced resistance to *Xoo* PR6. The *NPR1*-enhanced resistance phenotype segregated among the T_1 plants tested. Figure 1(b) presents inoculated leaf samples of six resistant T_1 plants from three *NPR1* T_0 lines (1, 2 and 6), along with TP309 and an *Xa21* transgenic line for comparison. Leaves were collected 2 weeks after inoculation. The six T_1 *NPR1* plants exhibited reduced lesion lengths compared to TP309, confirming the inheritance of the enhanced resistance phenotype. Lesions on susceptible plants, such as TP309, normally develop evenly down the leaf or mainly in the central vein of the leaf, causing great damage or death to the leaf. The lesions on *NPR1* plants tend to be restricted to the sides of the inoculated leaf, leaving the central vein relatively unaffected and resulting in better survival of the leaf. Thus the degree of enhanced resistance by *NPR1* over-expression appears to be greater than that measured directly by lesion length difference. However, the resistance resulting from *NPR1* over-expression is not as effective against *Xoo* PR6 as that from the *Xa21* resistance gene, as indicated by the shorter leaf lesion lengths of *Xa21* plants.

We conducted genomic DNA blot hybridization and polymerase chain reaction (PCR) analyses to confirm the presence of the *NPR1* transgene in the transgenic plants. Figure 2(a) shows results of genomic DNA blots of 17 independent *Ubi-NPR1* lines. The *NPR1* transgene is detected in all lines except the TP309 control. The different patterns of bands in different lines confirm that the transformed lines are independently derived. We also performed RNA blot hybridization to analyze the correlation between *NPR1* mRNA expression and the enhanced resistance phenotype. Figure 2(b) shows DNA and RNA blot hybridization results, and disease resistance rating of segregating T_1 plants from lines 1 (1-1, 1-9, 1-10 and 1-13), 2 (2-2, 2-3, 2-4 and 2-5), and 6 (6-1 to 6-8). To detect the transgene, PCR was utilized on progeny of lines 1 and 2, and genomic DNA blotting was done on progeny of line 6. The progeny of lines 1 and 2 segregate for the presence or absence of the *NPR1* transgene, as demonstrated by the absence of the transgene in 1-9, 1-10 and 2-3, and its presence in the others. All progeny from line 6 carry the transgene; however, a DNA polymorphism is observed in 6-5 and 6-8 that are both missing the lower band on the DNA blot, indicating that line 6 carries multiple insertions. Total RNA from these T_1 progeny, TP309, and *Arabidopsis* Col-0 was hybridized with *NPR1*. Progeny 1-9, 1-10 and 2-3

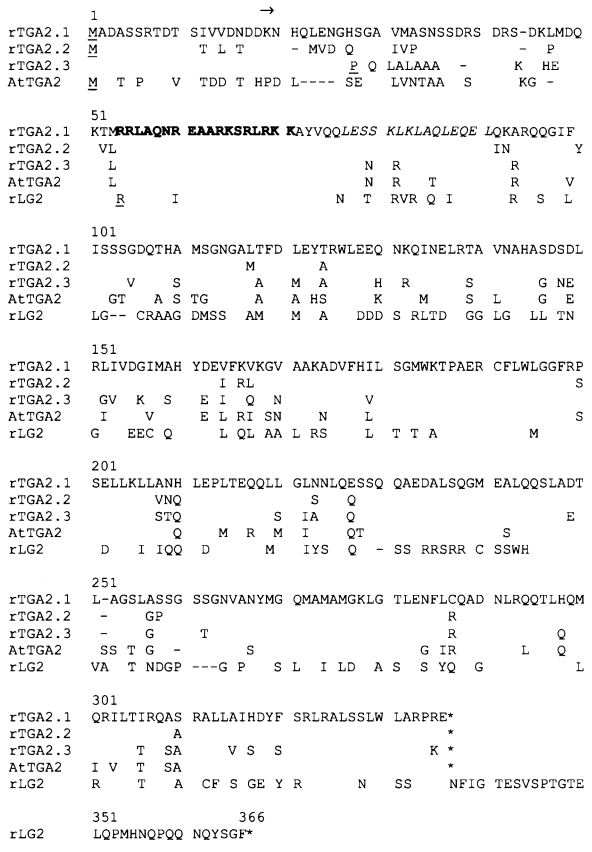

Figure 3. Amino acid sequence alignment of rTGA2.1, rTGA2.2, rTGA2.3, rLG2 and *Arabidopsis* TGA2.

Amino acid sequences of the four rice proteins and *Arabidopsis* (At) TGA2 are aligned using the PILEUP program of the Genetics Computer Group. Amino acid sequences identical to those of rTGA2.1 are left blank except for the first amino acids and the stop codons. The first amino acid of each protein is underlined, and the stop codon shown as an asterisk. – denotes a gap in sequence lineup. The basic region of rTGA2.1 is highlighted in bold and the leucine zipper region in italic. The sequence of the rTGA2.1 two-hybrid clone begins at amino acid K as indicated by the arrow. The first 18 amino acids of rTGA2.1 were obtained by PCR as described under Experimental procedures.

have no *NPR1* mRNA detected on RNA blots, consistent with the absence of the transgene. When inoculated with *Xoo* PR6, the enhanced resistance phenotype co-segregates with the presence of *NPR1* mRNA, indicating that the transgene confers the phenotype. In addition, when present, the *NPR1* mRNA levels (see 2-2, 2-4, 2-5, 1-1 and 1-13) are higher than that of *Arabidopsis* RNA. It should be noted that this *Arabidopsis* RNA sample is extracted from leaves heavily treated with 1 mM BTH to elevate *NPR1* mRNA levels; that the *NPR1* mRNA in untreated *Arabidopsis* leaf RNA is barely detectable in the same conditions (not shown); and that the weak 25S rRNA signal in the *Arabidopsis* lane is due to the use of a heterologous probe (rice 25S rDNA) and does not represent less RNA in the lane. Ryals *et al*. (1997) showed that BTH treatment induced *NPR1* mRNA accumulation by several-fold. Thus the *NPR1* mRNA level in the *Arabidopsis* sample in Figure 2(b) should be considered an elevated level. We regard any *NPR1* mRNA levels equal to or higher than this induced *Arabidopsis* level as a 'high level' of expression. Although the eight progeny of line 6 all contain the *NPR1* transgene, they express very different levels of *NPR1* mRNA. The *NPR1* mRNA levels cannot be correlated with copy number of the transgene. Lines 6-1, 6-2, 6-4, 6-6 and 6-7, which carry enhanced resistance and are rated R (lesion lengths 6 cm or shorter), display high levels of *NPR1* mRNA, whereas lines 6-3 and 6-5, which are fully susceptible and hence rated S (lesion lengths 9 cm or longer), have very low levels of *NPR1* mRNA. Line 6-8 has an *NPR1* mRNA level higher than 6-3 and 6-5, though lower than the *Arabidopsis* level and the other progeny of the same line, and displays enhanced resistance on bacterial inoculation. Thus mere expression of the *NPR1* transgene may not confer enhanced resistance, as in the cases of lines 6-3 and 6-5. The results for 6-8 suggest that a threshold level of the steady-state *NPR1* mRNA is required for enhancing resistance to *Xoo* PR6. It remains unclear what causes the difference in the steady-state *NPR1* mRNA levels in the progeny of the same line. However, these data clearly indicate that over-expression of *NPR1* leads to enhanced resistance to *Xoo* PR6.

To date, we have characterized a large number of T_2 plants from these *NPR1* transgenic lines, and the enhanced resistance phenotype was inherited to their T_2 progeny. Thus the *Ubi-NPR1* transgene is clearly not silenced through three generations. No obvious morphological changes were observed in the progeny of these transgenic lines.

Rice proteins that interact with NPR1 *are identified*

Since *Arabidopsis NPR1* can enhance disease resistance in rice, we reasoned that the components involved in the signal transduction pathway might be conserved in rice and *Arabidopsis*. To search for rice proteins interacting with NPR1, we used the yeast two-hybrid genetic method (Fields and Song, 1989). We generated a construct fusing the full-length *Arabidopsis* NPR1 to GAL4 DNA-binding domain (BD) and used it as bait to screen a rice cDNA library. After screening approximately 20 million yeast colonies transformed with library DNA, we recovered approximately 150 colonies that were both histidine-prototrophic and β-galactosidase-positive. When plasmid DNA was isolated and reintroduced into yeast, most of the tested clones retained the ability to confer the phenotype in a background containing BD::NPR1, but not BD alone. Sequence results of the positive clones place them in four different groups. Here we report on the first group, which

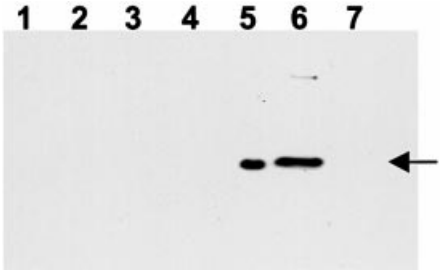

Figure 4. Immunoblot of NPR1 pulled down by rTGA2.1.
Baculovirus-expressed protein containing NPR1 was mixed with (6×) histidine-tagged rTGA2.1 or (6×) histidine-tagged GFP, or buffer. The protein complexes were pulled down with Ni-NTA-agarose resin. Protein eluted from the pellet was precipitated with ammonium sulfate, blotted to a membrane after electrophoresis, and probed with an anti-NPR1 antibody targeting the C-terminus of NPR1. All lanes are pull-down products except lane 5, which contains NPR1 loaded directly to serve as a size marker. Lane 1, no protein added, buffer only; lane 2, NPR1 only; lane 3, GFP only; lane 4, rTGA2.1 only; lane 6, NPR1 + rTGA2.1; lane 7, NPR1 + GFP. Arrow indicates location of NPR1 band.

contains four members belonging to the bZIP family of transcription factors.

These rice bZIP proteins share high degrees of sequence similarity with the *Arabidopsis* TGA2 protein, which has recently been shown to interact with NPR1. Figure 3 shows an amino-acid alignment of the *Arabidopsis* TGA2 protein and the four rice bZIP proteins, here named rTGA2.1, rTGA2.2, rTGA2.3 and rLG2. The 5′-end cDNA sequence encoding the first 18 amino acids of the rTGA2.1 protein was obtained by PCR amplification using the library DNA as template. The same PCR approach verified that the rTGA2.2 two-hybrid clone contained the full-length open reading frame. Thus in Figure 3, the rTGA2.1 and rTGA2.2 proteins are full length, while rTGA2.3 and rLG2 are apparently truncated proteins. The rTGA2.1, rTGA2.2 and rTGA2.3 proteins share 75, 76 and 78% overall identity, and 81, 82 and 82% similarity, respectively, with *Arabidopsis* TGA2. Although the homology is spread throughout the entire proteins, some regions have higher levels. For example, the basic regions of rTGA2.1, rTGA2.2, rTGA2.3 and *Arabidopsis* TGA2 share identical amino acids (in bold in Figure 3). The rTGA2.1 and rTGA2.2 gene products are 334 and 333 amino acids long, respectively, and are very similar in size to *Arabidopsis* TGA2 which contains 330 amino acids. The rLG2 protein is most similar to the gene product of maize *liguleless2* (*LG2*), which is involved in the establishment of the leaf blade–sheath boundary (Walsh *et al.*, 1998). Maize *LG2* encodes a protein 531 amino acids in length. rLG2 shares 81% sequence identity and 88% similarity with maize LG2, and only 57% identity and 66% similarity with *Arabidopsis* TGA2. Thus the four rice bZIP proteins that interact with NPR1 are from two subclasses: rTGA2.1, rTGA2.2 and rTGA2.3 fall in the TGA2 class; and rLG2 belongs to the LG2 class.

RTGA2.1 and NPR1 interact in vitro

We conducted *in vitro* protein–protein interaction experiments to confirm the binding between NPR1 and the rTGA2.1 protein. A (6×) histidine-tagged full-length rTGA2.1 protein was over-expressed in *Escherichia coli* using the pET15b expression system and purified by using Ni-NTA-agarose resin. To serve as a control, a (6×) histidine-tagged green fluorescence protein (GFP5) derived from jellyfish (Haseloff *et al.*, 1997) was also expressed and purified by the same way. The purified rTGA2.1 and GFP were mixed with a baculovirus-expressed protein extract containing NPR1 (Zhang *et al.*, 1999). The protein complexes were pulled down using Ni-NTA-agarose resin targeting the (6×) histidine tag. The presence of NPR1 was detected by antiserum raised against the C-terminus of NPR1 (Zhang *et al.*, 1999). Figure 4 shows results of the pull-down experiment. The anti-NPR1 antiserum detected the presence of NPR1 protein on the immunoblot only in the reaction containing NPR1 + rTGA2.1 (lane 6), but not in reactions containing either NPR1 + GFP (lane 7), rTGA2.1 only (lane 4), GFP only (lane 3), or NPR1 only (lane 2). These results show that the rice rTGA2.1 protein interacts specifically *in vitro* with NPR1.

RTGA2.1 binds to an SA-responsive TGA element and the rice RCH10 promoter in vitro

We tested the ability of the recombinant rTGA2.1 protein to bind to a SA-responsive TGA binding site. A 32 bp double-stranded oligonucleotide corresponding to the *Arabidopsis PR1* promoter (from –656 to –625), containing a sequence with the TGACG core required for SA-responsiveness, was synthesized and labeled as the probe for the gel mobility shift assay. As shown in Figure 5(a), a single band corresponding to protein–DNA complex is present (lane 3) when the rTGA2.1 protein is included. This band is absent when the GFP protein is used instead (lane 2), indicating that this complex is indeed the rTGA2.1–DNA complex and not a result from *E. coli* contamination proteins. Cold double-stranded oligonucleotides were included as competitors in the subsequent binding reactions to test the specificity of the protein–DNA interaction. The protein–DNA complex was competed away when excess (5×, 10× and 50×, from lanes 4–6) wild-type (W) oligonucleotide was included. When the same excess amounts of mutant (M) oligonucleotide that carries a 4 bp change in the center of the TGACGT consensus was added, it failed to compete away the complex (lanes 7–9). These data indicate that rTGA2.1 binds to the SA responsive TGA element from the *Arabidopsis PR1* promoter with high affinity and specificity.

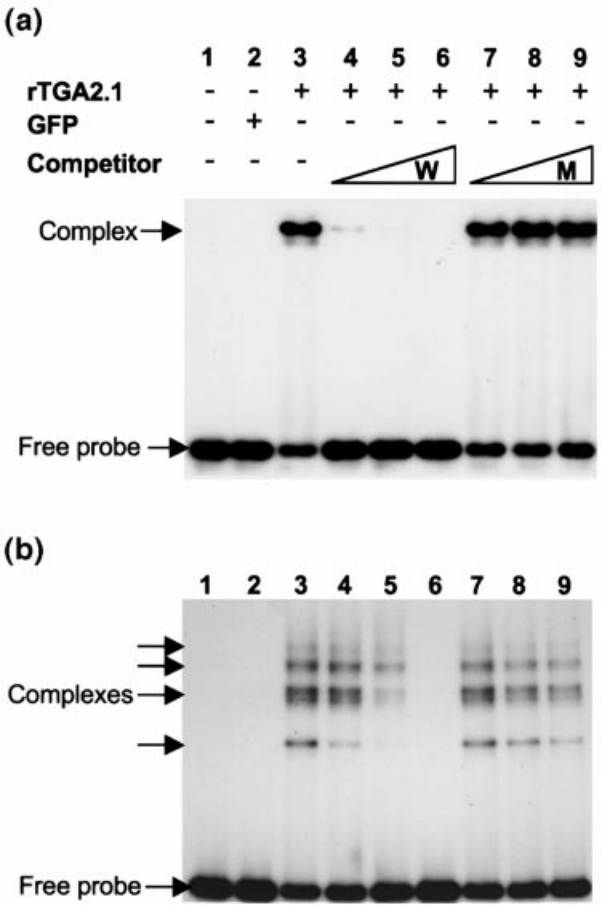

Figure 5. Gel mobility shift assay with rTGA2.1.
(a) An SA-responsive element (SARE) from the *Arabidopsis PR-1* promoter. The ^{32}P-labeled double-stranded SARE probe (corresponding to –656 to –625 of *PR-1* promoter) was incubated with rTGA2.1 (lanes 3–9), GFP (lane 2), or buffer (lane 1) before electrophoresis. For the competition experiment, rTGA2.1 was first mixed with cold wild-type (W) SARE or mutant (M) DNA competitor before incubation with the probe. The amounts of competitors were approximately 5×, 10× and 50× (left to right, indicated by triangles), respectively, in excess of the probe. Locations of the free probe and protein–DNA complex are indicated by arrows. Sequences of the wild type and mutant competitors are CTATTTTACTTACGTCATAGATGTGG and CTATTTTACTTAGTCGATAG-ATGTGG, respectively.
(b) Rice RCH10 promoter. A DNA fragment containing the proximal RCH10 promoter (from –232 to –14) was end-labeled as probe. The protein and competitor compositions of lanes 1–9 are the same as in (a), except for the concentration of competitors. The amounts of competitors from left to right were approximately 6×, 18× and 60×, respectively, of the probe.

We then tested binding of the rTGA2.1 protein to a rice RCH10 promoter element (from –232 to –14). The rice RCH10 gene encodes a basic chitinase and is induced by a fungal cell-wall elicitor in suspension-cultured cells (Zhu and Lamb, 1991). Figure 5(b) shows that rTGA2.1 (lane 3) but not GFP (lane 2) binds to the RCH10 probe, forming several bands of protein–DNA complexes, presumably due to the presence of multiple binding sites. When cold competitors (as above) were included in increasing amounts (6×, 18× and 60×, respectively, from left to right), the wild type (lanes 4–6) competed away the protein–DNA complexes effectively at 18× and 60×, whereas the mutant competitor (lanes 7–9) had little effect even at 60× excess amount. These results indicate that rTGA2.1 protein binds to the proximal promoter of RCH10, an elicitor-inducible rice gene, in a sequence-specific manner.

The npr1-1 and npr1-2 mutations affect the NPR1–rTGA interaction

Several npr1 and nim1 mutations that affect SAR in response to induction by SA have been shown to impair the interaction of NPR1 with *Arabidopsis* TGA proteins (Despres *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000). We have assessed the effects of npr1-1, npr1-2 and nim1-4 mutations on the interaction in the yeast two-hybrid system with rice proteins rTGA2.1, rTGA2.2, rTGA2.3 and rLG2, respectively. The npr1-1 mutation changes the highly conserved histidine-334 in the ankyrin domain to a tyrosine residue; the npr1-2 mutation carries a cysteine-to-tyrosine change at amino acid 150 (Cao *et al.*, 1997); nim1-4 has a conservative mutation changing arginine-432 to lysine (Ryals *et al.*, 1997). Wild-type NPR1, npr1-1, npr1-2 and nim1-4 proteins were produced in yeast cells in fusion to the LexA DNA-binding domain. The expression levels of these fusion proteins in yeast were monitored by immunological blot analysis using an anti-LexA monoclonal antibody (mAb). As shown in Figure 6, the anti-LexA mAb detected a 90 kDa band (arrow) corresponding to the predicted size of LexA::NPR1 fusion protein in all samples containing the LexA fusion proteins (lanes 2–5), but not in the one containing the LexA DNA-binding protein alone. With equal amounts of total protein loaded in each lane, the intensity of the LexA::NPR1 band was approximately equal to or lower than that of LexA::npr1-1 and LexA::npr1-2, and significantly lower than that of LexA::nim1-4. Thus npr1-1, npr1-2 and nim1-4 proteins, when fused to the LexA DNA-binding domain, are expressed and are at least as stable as the LexA::NPR1 protein in yeast cells.

The protein–protein interactions were first tested on leucine (Leu) dropout plates for prototrophy of the yeast cells and on X-gal plates for β-galactosidase (β-gal) activity. Figure 7(a) shows the arrangement of the different bait constructs on plate. The prey construct encoding the rice bZIP protein fused to the transcription activation domain present on each plate is indicated on the left of Figure 7(b,c). Figure 7(b) shows the results of the Leu dropout test, and Figure 7(c) displays the results of on-plate β-gal activity assay on yeast cells carrying combinations of different bait and prey constructs. Yeast cells containing NPR1 grew well on Leu dropout medium in combination with all four rice bZIP proteins (Figure 7b). Similarly, they

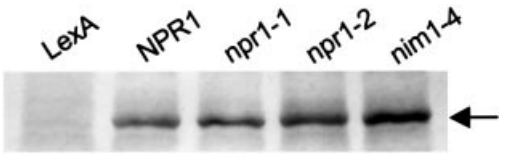

Figure 6. Immunoblot of LexA fusion proteins expressed in yeast.
Total protein extracts from yeast cells expressing NPR1, npr1-1, npr1-2 or nim1-4 as a protein fused to the LexA DNA-binding domain, or the LexA protein alone, were blotted on a membrane after electrophoresis and probed with an anti-LexA DNA-binding domain antibody. Approximately 40 μg protein was used for each lane. The location of the LexA::NPR1 protein is indicated by an arrow.

turned blue on medium containing the substrate X-gal (Figure 7c). While NPR1 + rTGA2.1 and NPR1 + rTGA2.2 gave darker blue colors, NPR1 + rTGA2.3 resulted in a lighter blue. Yeast cells carrying either the npr1-1 or npr1-2 fusion protein, or the LexA protein alone, failed to grow on the Leu dropout medium. These yeast cells also consistently remained white in the presence of X-gal, showing undetectable levels of β-gal activity, except for LexA + rTGA2.3 which gave faint bluish colonies. The nim1-4 containing yeast cells grew as well on Leu dropout medium, and were as blue as yeast cells containing NPR1. These results indicate that the npr1-1 and npr1-2 mutations abolish the interaction with the rice bZIP factors, and that the nim1-4 mutation has no effects.

A quantitative β-gal activity assay was also conducted using ONPG as the substrate. In Table 1, β-gal enzyme activity is presented as units; fold of induction due to the presence of NPR1 or npr1/nim1 is also calculated by dividing the number of units with the activity given by the LexA protein alone. In the absence of any NPR1 fusion protein, rTGA2.1, rTGA2.2 and rLG2 gave lower background levels of activity (0.5–0.6 units) than rTGA2.3 (1.9 units). In the presence of NPR1, interaction with the four rice bZIP proteins resulted in similar levels of absolute β-gal activity (ranging from 23 to 29 units). When the individual background activity is taken into consideration and fold of induction is calculated, rLG2 yields the highest number (58.6×) and rTGA2.3 gives the lowest number (13.2×) of induction; rTGA2.1 and rTGA2.2 have comparable numbers (43.7× and 45.4×). These differences should be interpreted carefully, as the amount of each bZIP protein was not determined.

Compared to the wild-type NPR1, the npr1-1 and npr1-2 mutations reduce interactions with all the rice bZIP proteins based on β-gal activity. The npr1-1 and npr1-2 mutations have stronger effects on reducing the interaction with rTGA2.3 and rLG2 (causing 11- to 29-fold reduction in β-gal activity) than with rTGA2.1 and rTGA2.2 (exhibiting six- to ninefold reduction). In summary, these data suggest that the NPR1 and rice bZIP protein interaction in yeast is greatly affected by the npr1-1 and npr1-2 mutations. The reduction in β-gal activity is not due to instability of the npr1-1 or npr1-2 protein as NPR1, npr1-1 and npr1-2 proteins were present in yeast cells at comparable levels, as detected by the immunoblot analysis (Figure 6). In contrast to npr1-1 and npr1-2, the nim1-4 mutation yields slightly higher β-gal activity compared to NPR1. However, the higher amount of the nim1-4 protein in yeast cells can account for this mild increase in β-gal activity. Thus the nim1-4 mutation appears to have no effects on the interaction of NPR1 with the rice bZIP proteins in the yeast two-hybrid assay.

Table 1. β-galactosidase (β-gal) activity induced by the interaction between bait and prey proteins in the yeast two-hybrid

		β-gal activity	
Prey[a]	Bait[b]	Units[c]	Fold of induction[d]
rTGA2.1	Vector	0.6 ± 0.2	
	NPR1	26.2 ± 10.3	43.7×
	npr1-1	3.4 ± 0.5	5.7×
	npr1-2	2.9 ± 0.2	4.8×
	nim1-4	37.6 ± 16.0	62.7×
rTGA2.2	Vector	0.5 ± 0.1	
	NPR1	22.7 ± 10.1	45.4×
	npr1-1	2.8 ± 0.2	5.6×
	npr1-2	3.7 ± 0.8	7.4×
	nim1-4	27.1 ± 4.0	54.2×
rTGA2.3	Vector	1.9 ± 0.8	
	NPR1	25.1 ± 4.1	13.2×
	npr1-1	1.0 ± 0.1	0.5×
	npr1-2	2.3 ± 0.3	1.2×
	nim1-4	23.6 ± 6.5	12.4×
rLG2	Vector	0.5 ± 0.1	
	NPR1	29.3 ± 9.8	58.6×
	npr1-1	1.0 ± 0.3	2.0×
	npr1-2	1.4 ± 0.9	2.8×
	nim1-4	31.0 ± 6.6	62.0×

[a]The prey protein is fused to a transcription activation domain sequence encoded in the pB42AD vector.
[b]The bait protein is either the LexA DNA-binding domain alone (vector) or fused to the LexA DNA-binding domain encoded in the pJK202 vector.
[c]Each value represents the average ± SD of three colonies. Each colony was assayed three times.
[d]Fold of induction is obtained when the individual number of units is divided by the background value with empty vector.

Discussion

Transgenic rice plants over-expressing the *Arabidopsis NPR1* gene display enhanced resistance to rice bacterial blight. This is the first case in which the *Arabidopsis NPR1* introduced into another species has been demonstrated to enhance resistance to a disease, and is also the first example where a dicot SAR regulatory gene has been

utilized in a monocot to achieve enhanced disease resistance. These results may greatly facilitate the task of battling diseases of other cereals. Moreover, a threshold level of *NPR1* mRNA is required for the enhanced resistance phenotype in the transgenic plants. These results are in line with previous reports showing the correlation between disease resistance and *NPR1* expression levels in *Arabidopsis* (Cao *et al*., 1998).

Furthermore, using NPR1 as bait in a yeast two-hybrid screen, we have isolated four rice cDNA clones, *rTGA2.1, rTGA2.2*, *rTGA2.3 and rLG2*, encoding proteins that belong to the family of bZIP transcription factors. The rTGA2.1, rTGA2.2 and rTGA2.3 proteins share 75 to 78% identity to the *Arabidopsis* TGA2 protein which was previously shown to interact with NPR1. The interaction between rTGA2.1 and NPR1 was confirmed using *in vitro* pull-down experiments. The basic regions of rTGA2.1, rTGA2.2, rTGA2.3 and *Arabidopsis* TGA2 are completely conserved. There are regions besides the basic domain that share particularly high levels of identity, although the similarity extends throughout the whole protein. These regions may be involved in receiving signals by interacting with other proteins, such as NPR1 and an inhibitor implicated in regulating the activity of SARP (Jupin and Chua, 1996).

As the basic region of bZIP protein is directly involved in contacting DNA (Ellenberger *et al*., 1992), these rice and *Arabidopsis* proteins may recognize very similar DNA sequences. We have shown that the rTGA2.1 protein, similar to the *Arabidopsis* TGA2, binds to the SA-responsive element of the *Arabidopsis PR-1* promoter in a sequence-specific manner. Similarly, rTGA2.1 also binds sequence-specifically to the proximal promoter of the rice RCH10 gene, which is induced by a fungal elicitor. These results support the notion that the rice TGA proteins may be involved in NPR1-mediated induction of rice defense genes, such as RCH10.

The rLG2 protein is most similar to the gene product of maize *LG2* (81% identity), and less similar to *Arabidopsis* TGA2 (57% identity). Maize LG2 contains 531 amino acids and is significantly larger due to the presence of a long N-terminal region located before the bZIP domain. The maize *LG2* gene is involved in the establishment of the leaf blade–sheath boundary and functions during the transition from the vegetative to the reproductive shoot apex (Walsh and Freeling, 1999). Therefore rLG2 belongs to a subclass of plant bZIP transcription factors that may differ from the TGA2 group, not only in size but also in the biological functions in which they are involved. In consideration of these results, we do not exclude the possibility that overexpression of *NPR1* in rice may lead to subtle developmental changes, even though we have not observed obvious developmental abnormality in these rice plants.

The results of on-plate and quantitative β-gal activity assays suggest that rLG2 may bind to NPR1 as strongly as rTGA2.1, although the expression levels of these proteins have not been analyzed. However, the rLG2 clone used in these assays is apparently lacking its N-terminal portion of the protein. It is possible that the N-terminal portion of rLG2 missing in our clone may affect its interaction with NPR1, as Zhou *et al*. (2000) show that the N-terminal portion of TGA1 affects interaction with NPR1.

NPR1, npr1-1 and npr1-2 fused to LexA have comparable protein levels, whereas the nim1-4 protein has a higher level. The nim1-4 mutation has little effect on the interaction of NPR1 with the rice bZIP proteins. On the contrary, compared with wild-type NPR1, both npr1-1 and npr1-2 reduce the NPR1–bZIP interaction. The npr1-1 mutation is located in the ankyrin domain, npr1-2 in the N-terminal part of the protein (Cao *et al*., 1997), and nim1-4 in the C-terminal portion after the ankyrin repeats (Ryals *et al*., 1997). These data are consistent with previous reports (Zhang *et al*., 1999) showing that only the N-terminal two-thirds of NPR1, including the ankyrin repeats, are required for high-affinity interaction with *Arabidopsis* TGA proteins. The fact that nim1-4 fails to affect the NPR1–TGA interaction suggests that this mutation may abolish NPR1/NIM1 function in *Arabidopsis* by a mechanism different from that of npr1-1, npr1-2 and other mutations that impair NPR1–TGA interaction. It is likely that the nim1-4 mutation may affect NPR1/NIM1 interaction with other unidentified SAR signaling proteins. Another possibility is that nim1-4 might affect RNA splicing, as the point mutation is located only three nucleotides upstream of a splicing site (Ryals *et al*., 1997). In summary, these rice TGA proteins interact with NPR1 in a way similar to the *Arabidopsis* TGA proteins in the yeast two-hybrid system. Taken together, these results support the notion that rice shares with *Arabidopsis* a similar disease-resistance pathway mediated by *NPR1*. We have isolated an NPR1 homolog from rice that also interacts with rTGA2.1 (M.-S. Chern and P.C. Ronald, unpublished results) in the yeast two-hybrid system. Thus it appears not only that *NPR1* functions in rice to enhance disease resistance, but also that it may function by a similar molecular mechanism as in *Arabidopsis*.

However, the question still remains how rice maintains the high levels of endogenous SA (Silverman *et al*., 1995) without activating the resistance response. One possibility is that the regulated points in the pathways might be different in rice than in *Arabidopsis*, even though the components that constitute the signal transduction pathways are similar. Yu *et al*. (1997) report that, in potato, a high basal level of SA does not lead to constitutive resistance, and the development of SAR against *Phytophthora infestans* may involve increased sensitivity to SA. Similarly, a postulated SA receptor might be further controlled in rice by another signal, such as protein phosphorylation, whereas it might need only SA to activate it in *Arabidopsis*. Another possibility is that, in rice, SA is

localized in a different cellular compartment than in *Arabidopsis*. Alternatively, an SA derivative, instead of SA itself, might be the active molecule for signaling in rice. This view is consistent with previous reports that BTH, a SA analog, induces disease resistance in rice (Schweizer *et al.*, 1999). Thus it seems likely that regulation of *NPR1*-mediated resistance in rice differs from that in *Arabidopsis*.

The fact that the SAR regulatory gene *NPR1* can enhance resistance to the rice bacterial blight pathogen *Xoo* indicates that an *NPR1* counterpart in rice may be involved in the signal transduction of R genes (e.g. *Xa21*) conferring resistance to bacterial blight. Experiments are under way to address the possible involvement of the rice *NPR1* homolog in the resistance-signaling pathway of *Xa21*. The levels of resistance to rice bacterial blight mediated by the over-expressed NPR1 did not reach the level conferred by the *Xa21* resistance gene. This result may be due to functional differences between the *Arabidopsis NPR1* and the rice counterpart, or to a high turnover rate of *NPR1* mRNA in rice. In addition, NPR1 may not be the sole signaling protein controlling *Xa*21-conferred resistance.

In summary, we have shown that *NPR1* works in rice to enhance resistance, and have uncovered several rice components that are probably involved in an *NPR1*-mediated signal transduction. These findings should greatly facilitate study on disease resistance pathways in rice and other monocots, and may have a profound impact on controlling diseases in economically important crops such as cereals.

Experimental procedures

Plant materials and growth conditions

Rice (*Oryza sativa*) seedlings were grown in the greenhouse until ready for *Xanthomonas* inoculation. For bacterial inoculation, 6-week-old plants were transferred to growth chambers and grown on a 14 h daytime period and a 28/26°C cycle. Bacterial blight inoculation was done with Philippine race 6 strain PXO99 (provided by J. Leach, Kansas State University) using the scissors-dip method (Kauffman *et al.*, 1973). For rice transformation, the *Agrobacterium tumefaciens* strain EHA105 was utilized to infect callus induced from mature embryos of cultivar Taipei 309, according to a transformation protocol (Cheng *et al.*, 1997) modified from Hiei's method (Hiei *et al.*, 1994).

Plasmid construction for rice transformation

The pCambia 1301 binary vector (http://thaliana.botany.wisc.edu/cambia/index.html) was used as the backbone to construct plant expression vectors. A 2 kb DNA fragment containing the maize *ubiquitin* promoter (Christensen and Quail, 1996) was cut out from the pAHC17 plasmid with *Hin*dIII + *Bam*HI and cloned into a vector composed of pCambia 1301 plus a *nos* 3′ sequence (C1301Nos3′), yielding plasmid Ubi-C1301. Similarly, a 35S-C1301 vector was constructed by subcloning a 0.9 kb fragment carrying the CaMV *35S* promoter into the C1301Nos3′ vector using *Pst*I and *Kpn*I sites. In order to generate restriction sites for subcloning the *Arabidopsis thaliana NPR1* cDNA into the Ubi-C1301 and 35S-C1301 vectors, an NPR1/SK plasmid was created by cloning a 2 kb *NPR1* cDNA fragment from plasmid pKEX to pBlueScript SK-via the *Eco*RI and *Sac*I sites. The *NPR1* cDNA was then cut out from NPR1/SK and cloned into 35S-C1301 using *Cla*I and *Sac*I to create plasmid *35S-NPR1*(C1301). The cDNA was also excised from NPR1/SK with *Eco*RV and *Sac*I, and inserted into Ubi-C1301 to generate plasmid *Ubi-NPR1*(C1301). Transformation of *Agrobacterium* EHA105 with plasmids *Ubi-NPR1 and 35S-NPR1* was done by electroporation according to the manufacturer's instructions, using the Cell-Porator (Life Technologies, Rockville, MD, USA).

PCR, DNA and RNA blot hybridization

Rice genomic DNA extraction was done according to a previously described protocol (Dellaporta *et al.*, 1984). Total RNA extraction followed the manufacturer's instructions using the Trizol reagent (Life Technologies) with some modifications: 3 ml of reagent was used for each gram of leaf tissue. Total RNA was further purified by precipitating the RNA in 2 M LiCl (Sambrook *et al.*, 1989). PCR of the *NPR1* transgene used primers Npr1-1 (GAACCCGGG-ATGGACACCACCATTG) and Npr1-7 (AAGGATCCTCAAGGT-ACCTCCAAACCAAG). DNA and RNA blotting and hybridization were done essentially as described by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using Hybond-N+ membrane, except that RNA hybridization was conducted at 42°C with the addition of 50% formamide and 10% dextran sulfate in the hybridization solution.

Yeast two-hybrid screening and isolation of cDNA 5′ ends

The rice cDNA expression library was prepared (Wen-Yuan Song and Pam Ronald, unpublished data) in the pAD-GAL4 prey vector (Stratagene, La Jolla, CA, USA) using leaf tissue of IRBB21 inoculated with *Xoo* PR6. To construct the NPR1 bait plasmid, an 800 bp 5′ DNA fragment was PCR amplified with primers Npr1-1 and Npr1-2 (TTCTGCAGTTACGAGACATGTTTCTTTAC). This fragment was digested with *Sma*I + *Kpn*I and ligated into a *Sma*I + *Sac*I-digested pMC86 vector, together with a 1.2 kb *Kpn*I–*Sac*I fragment containing the 3′ end of NPR1. The construct was confirmed by sequencing. The pMC86 bait vector was constructed by swapping the GAL4 transcription activation-domain sequence of pPC86 (Chevray and Nathans, 1992) with the GAL4 DNA binding-domain sequence of pPC97 using the *Kpn*I and *Sac*I sites. The pMC86 bait plasmid carries the TRP1 selection marker and retains the multiple cloning sites of pPC97 for fusion to the GAL4 DNA-binding domain. The yeast strain HF7c (Clontech, Palo Alto, CA, USA) was used for cDNA library screening. Yeast transformation and selection was conducted essentially as recommended by the manufacturer (Clontech).

The 5′-end of rTGA2.1 cDNA was amplified from the same cDNA library by two sequential PCRs: first with primers ADR1 (ACCGGGAGAGATCGAATTCGGCACGA) and mn1-1 (GAAGCC-ATGACTGCACCA); then with primers ADR1 and mn1-2 (CACCACTATGTCCGTTTTC). The full-length open reading frame of rTGA2.1 cDNA was assembled by performing two sequentially PCR amplifications with template MN1 and primer pairs mn1-3 (GACTGACACATCGATTGTTGTAGACAACGACGACAAAAACCAC-CAGTTAGA) + mn1-5 (AAAGGATCCTTACTCCCGTGGCCTAGC-AAG), then mn1-5 + mn1-4 (AAAGGATCCCATGGCAGATGCTA-GTTCAAGGACTGACACATCGATTG). Similarly, the 5′ end of

rTGA2.2 cDNA was amplified with primer pairs ADR1 + mn8-1 (TTATCGTCGGTATCCAGGA) and ADR1 + mn8-2 (GGACTGTTGATGTGTCAGT), sequentially. The sequence of the PCR amplified 5′ end of rTGA2.2 confirmed that the two-hybrid clone (MN8) already contains the complete open reading frame. DNA and protein sequences were analyzed using BLAST 2.1 and the BESTFIT AND PILEUP programs of the Wisconsin package version 10.1, Genetics Computer Group (Madison, WI, USA).

Protein expression in E. coli *and purification*

To express the rTGA2.1 protein in *E. coli*, the full-length cDNA was amplified by PCR sequentially, first using 5′ primer mn1-7 (AAAGGATCCCATGGCAGATGCTAGTTCAAGGA) and 3′ primer mn1-6 (ATCCTCCTCAGAAATCAGCTTTTGCTCCCGTGGCCTAGCAAG), then 5′ primer mn1-7 and 3′ primer (AAAGGATCCACTAGTTTACTTCTCCAGCAGATCCTCCTCAGAAATCAG), which also encodes a cMYC tag. The amplified cDNA was digested with *Bam*HI, cloned into pBlueScript SK-, and confirmed by sequencing. The insert was then subcloned in frame into the *Bam*HI site of the pET15b expression vector (Novagen, Madison, WI, USA), creating a (6×) His-rTGA2.1 fusion construct. For GFP plasmid construction, the GFP insert in the pBINm-gfp5-ER plasmid (Haseloff *et al.*, 1997) was cut with *Sac*I, blunt-ended by the exonuclease activity of T4 DNA polymerase, digested with *Eco*RI, and cloned into the *Eco*RI and *Sma*I sites of pBlueScript SK-. The GFP insert was excised from pBlueScript with *Xho*I and *Bam*HI and inserted into the pET15b vector digested with the same enzymes, yielding a (6×) His-GFP fusion construct. These plasmids were used to transform *E. coli* host BL21(DE3)pLysS. Induction of protein expression was carried out according to the manufacturer. Protein purification with Ni-NTA-agarose resin followed the manufacturer's recommendation (Qiagen, Chatsworth, CA, USA). In brief, the over-expressed recombinant protein was allowed to bind to an Ni-NTA-agarose resin, and eluted with 0.5 M of imidazole buffer after extensive step washes with 20, 50, 80 and 120 mM imidazole buffers. Eluted protein was dialyzed into a buffer containing 20 mM Tris pH 8.0 and 0.5 mM EDTA.

In vitro *protein pull down*

The *in vitro* protein–protein interaction was conducted in 100 μl volume by adding 2 μl (12 mg ml^{-1}) of baculovirus-expressed NPR1 (Zhang *et al.*, 1999), 12.6 μl (7.5 mg ml^{-1}) of purified rTGA2.1 protein, and enough wash buffer (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl and 20 mM imidazole) to make up the volume. The reaction was incubated on ice for 2 h. The reaction mixture was mixed with 25 μl Ni-NTA-agarose resin (Qiagen) and incubated for another 2 h on ice. The resin was spun down and rinsed five times, each time with 100 μl wash buffer. Protein pulled down by the resin was eluted twice, each time with 50 μl elution buffer (50 mM NaH_2PO_4 pH 8.0, 150 mM NaCl, 500 mM imidazole), and precipitated by bringing the solution to 60% saturation of ammonium sulfate. Protein precipitate was spun down, resuspended in 30 μl of SDS protein loading buffer, and run on an 8% polyacrylamide gel. After transfer to a membrane the blot was probed with an anti-NPR1 antibody (Zhang *et al.*, 1999).

Gel mobility shift assay with competitors

The procedure of probe preparation and gel mobility shift assay has been described previously (Chern *et al.*, 1996). The SARE probe (from –656 to –625 of *PR-1* promoter; Lebel *et al.*, 1998) containing LS7, which was identified as required for SA induction, was prepared by annealing oligonucleotides SARE-1 (CTATTTTACTTACGTCATAGATGTGG) and SARE-3 (ATGCCGCCACATCTATGACGTAAG). The double stranded oligo DNA was then labelled with ^{32}P-dCTP by using the Klenow enzyme to fill in the extrusions at both ends. The labeled probe was purified from a polyacrylamide gel. The wild-type competitor was prepared from annealing oligonucleotides SARE-1 and SARE-2 (CCACATCTATGACGTAAGTAAAATAG) and the mutant carrying a 4 bp change in LS7 was created from combining oligonucleotides SAREm-1 (CTATTTTACTTAGTCGATAGATGTGG) and SAREm-2 (CCACATCTATCGACTAAGTAAAATAG). The rice RCH10 promoter fragment from –232 to –14 relative to the transcription start site (Zhu and Lamb, 1991) was PCR-amplified from IR24 genomic DNA with primers rch10-21 (GGAAGCTTGAATTCAACCCCTTTTGCCCCTCTAAT) and rch10-22 (CCATGGGTCGACTGAGGAAGAAGCGATGGGAGC) and cloned into the pCR-Blunt-TOPO vector (Invitrogen, San Diego, CA, USA). The RCH10 insert was excised as a 287 bp DNA fragment with *Nco*I and *Xba*I, end-labeled with ^{32}P-dCTP, and purified in a polyacrylamide gel. The rTGA2.1 and GFP proteins used in the gel mobility shift assay are as described above.

Generation of npr1/nim1 mutants and test of interaction in yeast

The NPR1 cDNA was amplified with primers Npr1-15 (AGAGAATTCATGGACACCACCATTG) and Npr1-6 (AAGGATCCTGCAGCAATAATACAC) in order to generate cloning sites compatible with the two-hybrid vector. The npr1-1, npr1-2 and nim1-4 mutants were generated by PCR. To make npr1-1, two fragments containing the mutation were amplified using primers Npr1-15 and Npr1-12 (GCAGCAACATAAAGCACCGTATATCC) and primers Npr1-13 (TACGGTGCTTTATGTTGCTGCGATGC) and Npr1-6, respectively. The two fragments were denatured, re-annealed, and extended to yield full-length npr1-1; the full-length npr1-1 was amplified by PCR with primers Npr1-15 and Npr1-6. Similarly, to generate the nim1-4 mutant, primers Npr1-10 (GTGCAACCTTATTTTCAAGATCGAGCAG) and Npr1-11 (GATCTTGAAAATAAGGTTGCACTTGCTCAAC) were used in combination with primers Npr1-15 and Npr1-6. A partial clone of npr1-2 was created by PCR using primers Npr1-15 and Npr1-14 (AAGCCACGTGGCAGCAATTCTCGTCTGCGTATTCAGAAACTCCTTTAG); the PCR product was ligated to the rest of the cDNA sequence to yield full-length npr1-2 through a unique *Pml*I site in the two DNA fragments. The NPR1, npr1-1, npr1-2 and nim1-4 cDNA fragments were inserted in the pJK202 vector via *Eco*RI and *Bam*HI sites to create LexA fusion constructs.

The rTGA2.1, rTGA2.2, rTGA2.3, and rLG2 sequences were excised from the original clones (MN1, MN8, MN38 and MN140, respectively) pulled out in two-hybrid screening, and inserted in the pB42AD vector (Clontech) via *Eco*RI and *Xho*I sites to generate activation domain fusion constructs. EGY48 yeast cells were transformed with the prey and bait constructs simultaneously. The leucine dropout assay, the X-gal on-plate assay, and the ONPG (*o*-nitrophenyl β-D-galactopyranoside) β-gal quantitative assay were carried out according to Clontech's yeast protocols. For the β-gal quantitative assay, three yeast colonies were picked for each construct; three replicates of the reaction were performed for each colony. Total protein extracts were prepared from yeast cells using the TCA method (Clontech). Immunoblot analysis of

yeast protein was conducted with a monoclonal antibody against the LexA DNA-binding domain (Clontech).

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