The *Arabidopsis* *AtNPR1* Inversely Modulates Defense Responses Against Fungal, Bacterial, or Viral Pathogens While Conferring Hypersensitivity to Abiotic Stresses in Transgenic Rice

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The nonexpressor of pathogenesis-related (*PR*) genes (*NPR1*) protein plays an important role in mediating defense responses activated by pathogens in *Arabidopsis*. In rice, a disease-resistance pathway similar to the *Arabidopsis* *NPR1*-mediated signaling pathway one has been described. Here, we show that constitutive expression of the *Arabidopsis* *NPR1* (*AtNPR1*) gene in rice confers resistance against fungal and bacterial pathogens. *AtNPR1* exerts its protective effects against fungal pathogens by priming the expression of salicylic acid (SA)-responsive endogenous genes, such as the *PR1b*, *TLP* (*PR5*), *PR10*, and *PBZ1*. However, expression of *AtNPR1* in rice has negative effects on viral infections. The *AtNPR1*-expressing rice plants showed a higher susceptibility to infection by the Rice yellow mottle virus (RYMV) which correlated well with a misregulation of RYMV-responsive genes, including expression of the SA-regulated RNA-dependent RNA polymerase 1 gene (*OsRDR1*). Moreover, *AtNPR1* negatively regulates the expression of genes playing a role in the plant response to salt and drought stress (*rab21*, *salt*, and *dip1*), which results in a higher sensitivity of *AtNPR1* rice to the two types of abiotic stress. These observations suggest that *AtNPR1* has both positive and negative regulatory roles in mediating defense responses against biotic and abiotic stresses.

Additional keywords: *Erwinia chrysanthemi*, *Fusarium verticillioides*, *Magnaporthe oryzae*, and *Oryza sativa*.

Plant signaling pathways involved in the plant response to biotic and abiotic stresses are composed of intricate networks with frequent cross-talk that allow the plant to activate an appropriate spectrum of responses depending on the nature of the external stimuli. This leads to the production of proteins with direct roles in alleviating the damaging effects of stressful conditions. The plant response to pathogen attack includes the rapid generation of reactive oxygen species, changes in ion fluxes across the plasma membrane, cell wall reinforcement, activation of protein phosphorylation or dephosphorylation

cascades, production of antimicrobial compounds (i.e., phytoalexins), and accumulation of pathogenesis-related (*PR*) proteins (Hammond-Kosack and Parker 2003; van Loon et al. 2006). Moreover, resistance responses locally activated in primary pathogen-infected plant tissues are often extended to distant, noninfected tissues, a phenomenon which is referred to as systemic acquired resistance (SAR) (Ryals et al. 1996). Establishment of SAR is associated with the coordinated expression of SAR genes, many of these encoding *PR* proteins (Ward et al. 1991), as well as with the induction of the protein secretory pathway (Wang et al. 2005). In *Arabidopsis* and tobacco plants, salicylic acid (SA) is an essential signal molecule for SAR (Dempsey et al. 1999).

The nonexpressor of *PR* genes (*NPR1*) (also known as *nim1*, noninducible immunity) gene is a key regulator of SA-mediated SAR in *Arabidopsis* (Cao et al. 1994, 1997; Delaney et al. 1995; Glazebrook et al. 1996; Ryals et al. 1997; Shah et al. 1997). *NPR1* regulates expression of *Arabidopsis* defense genes by interacting with members of the TGA class of basic, leucine-zipper transcription factors (Despres et al. 2000; Zhang et al. 1999; Zhou et al. 2000). Redox changes in *Arabidopsis* cells regulate the functioning of *NPR1* (Mou et al. 2003). In noninduced cells, oxidized *NPR1* forms inactive oligomers that remain in the cytosol. SA application or exposure of the plant to the pathogen triggers a change in the molecular stage of *NPR1*, the *NPR1* protein being reduced from an inactive oligomeric complex to an active monomeric state. Monomeric *NPR1* is then translocated into the nucleus where it interacts with TGA factors for activation of *PR* gene expression (Despres et al. 2000; Kinkema et al. 2000; Rochon et al. 2006; Zhang et al. 2003).

It is also known that overexpression of the *Arabidopsis* *NPR1* (hereafter referred to as *AtNPR1*) gene in *Arabidopsis* results in enhanced resistance to bacterial and oomycete pathogens (Cao et al. 1998; Friedrich et al. 2001). When introduced into rice, *AtNPR1* confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern et al. 2001; Fitzgerald et al. 2004). Overexpression of the rice *AtNPR1* homolog, the *OsNPR1* gene (also named *NH1*), also enhances plant resistance to *X. oryzae* pv. *oryzae* in rice (Chern et al. 2005; Yuan et al. 2007). Together, these findings support the existence of a disease resistance pathway in rice similar to the *Arabidopsis* *NPR1*-mediated signaling pathway. Moreover, wheat and tomato plants expressing the *Arabidopsis* *AtNPR1* gene displayed enhanced resistance to fungal and bacterial diseases (Lin et al.

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2004; Makandar et al. 2006). More recently, overexpression of an *AtNPR1* homolog from apple (*MpNPR1-1*) in transgenic apple has been shown to confer increased resistance to a bacterial and two fungal pathogens (Malnoy et al. 2007). In other studies, overexpression of *OsNPR1* in rice results in enhanced herbivore susceptibility (Yuan et al. 2007). No information is currently available on the possible involvement of *AtNPR1* as a regulator of plant responses to abiotic stresses.

On the other hand, contradictory results are found in the literature on the possible involvement of *AtNPR1* in plant-virus interactions. Whereas *AtNPR1* appears not to be required for resistance to *Turnip crinkle virus* in *Arabidopsis* plants (Kachroo et al. 2000), the tobacco *NPR1* gene appears to be required for N-mediated resistance to *Tobacco mosaic virus* (TMV) (Liu et al. 2002). Concerning the plant response to viral infection, it is well known that RNA-dependent RNA polymerases (RDR or RdRP) are implicated in antiviral defenses. Plant RDR also participate in post-transcriptional gene silencing (PTGS) (Baulcombe 2004; Dalmay et al. 2000; Mourrain et al. 2000). Three functional RDR (RDR1, RDR2, and RDR6) have been identified in plants (Wassenegger and Krczal 2006). In *Arabidopsis*, RDR1 and RDR6 (also known as SDE1 and SGS2) are required in the cytoplasmatic RNA silencing pathway that silences transgenes and viruses. Moreover, the expression of the *Arabidopsis* and tobacco *RDR1* genes (referred as *AtRdRP1*, *NtRdRP1*, and *NbRdRP1*) has been described to be induced by treatment with SA and infection with certain viruses (Xie et al. 2001; Yang et al. 2004; Yu et al. 2003).

In this work, we generated transgenic rice lines constitutively expressing the *Arabidopsis AtNPR1* gene. The impact of transgenic expression of *AtNPR1* on the expression of stress-related rice genes as well as performance of *AtNPR1* rice plants grown under stress conditions, both biotic and abiotic conditions, was determined. We show that *AtNPR1* modulates the expression of genes involved in resistance to different types of fungal, bacterial, or viral pathogens. Whereas expression of *AtNPR1* in rice confers resistance to fungal and bacterial pathogens, *AtNPR1* rice shows higher susceptibility to infection by the *Rice yellow mottle virus* (RYMV). Finally, the expression of *AtNPR1* in rice notably interferes with the expression of salt and drought-associated genes, which results in hypersensitivity to these two types of abiotic stresses.

RESULTS

Phenotype of *AtNPR1* rice plants.

For expression of the *Arabidopsis AtNPR1* gene in transgenic rice, a pCAMBIA-derived construct containing the *AtNPR1* cDNA (Cao et al. 1997) under the control of the maize ubiquitin-1 (*ubi*) promoter was prepared. The elite japonica rice (*Oryza sativa* L.) cultivar was used in this study. Transgenic rice was produced by *Agrobacterium*-mediated transformation. Representative T0 events accumulating *AtNPR1* mRNA at different levels in their leaves were selected (Fig. 1A) and used as the parental lines to obtain T2 homozygous progeny plants. Northern blot analysis of selected *AtNPR1* rice plants confirmed stable expression and inheritance of the transgene for at least three generations (results not shown).

The developmental performance of *AtNPR1* rice plants grown in the greenhouse differed from that of control, nontransformed plants. Thus, transgenic *AtNPR1* rice plants grown in the greenhouse showed growth retardation and reduced height compared with wild-type plants (Fig. 1B). Moreover, seeds produced by *AtNPR1* plants were smaller than seeds produced by control plants (Fig. 1C). Other studies in *Arabidopsis* showed the effects caused by an alteration in the level of *AtNPR1* expression, or mutations that either prevent (*npr1* mutant) or cause a constitu-

tive expression (*cpr* mutants) of the inducible plant defense response on plant fitness (Heidel and Dong 2006; Heidel et al. 2004). These studies revealed that the precise fitness consequences of these mutations and transgenics were highly dependent on the environmental context. Although results here presented show that *AtNPR1* expression in rice has important consequences in terms of the “physiological costs” of the plant when growing under controlled conditions, an interpretation for these results in terms of the balance between fitness costs and disease resistance will require additional studies in plants growing under different environmental conditions.

When transferred to plant growth chambers, *AtNPR1* plants developed spontaneous lesions in their leaves evoking a lesion mimic phenotype (Fig. 2A). Lesions were visible as early as 2 to 3 days after transferring these plants from the greenhouse to the growth chamber. Transgenic *AtNPR1* rice lines maintained in the greenhouse did not exhibit lesions. The typical cellular markers of lesion formation, such as accumulation of superoxide (O_2^-) ions and autofluorescent material in the cell walls, were also produced in *AtNPR1* plants grown in the growth chamber (Fig. 2B and C). None of these histochemical markers (accumulation of superoxide and autofluorescent material) were detected in greenhouse-grown *AtNPR1* plants (data not shown). Together, these studies revealed that constitutive expression of *AtNPR1* in rice reduces growth and productivity in the greenhouse and, only in growth chambers, *AtNPR1* triggers development of spontaneous lesions. These results contrast with those obtained in *Arabidopsis* plants which exhibited no visible phenotypic alterations despite constitutive expression of the *AtNPR1* gene (Cao et al. 1998).

Disease resistance of *AtNPR1* rice plants to fungal and bacterial pathogens.

Transgenic *AtNPR1* plants were tested for resistance to infection with the fungal pathogens *Magnaporthe oryzae* and *Fusarium verticillioides*, the causal agents of the blast and bakanae diseases of rice. Four independently derived T2 homozygous lines were used in all the disease resistance assays carried out in this work (lines 1, 2, 13, and 24). For studies on blast resistance, leaves from *AtNPR1* and wild-type plants were inoculated with increasing doses of *M. oryzae* spores. Differences in the degree and development of disease symptoms caused by *M. oryzae* between *AtNPR1* and untransformed plants were clearly observed (Fig. 3A). Blast lesions developed actively in leaves of wild-type plants but not in leaves of *AtNPR1* lines. The ability of *AtNPR1* plants to block the in planta growth of *M. oryzae* was further assessed by measuring the average lesion size and by counting the number of spores produced in infected leaves of both control and transgenic lines (Fig. 3B and C).

Resistance of *AtNPR1* rice seedlings against the fungus *F. verticillioides*, a seedborne and soil-transmitted pathogen, was assayed. *F. verticillioides*-infected seed from the *AtNPR1* lines germinated and developed (Fig. 3D). In contrast, fungus-infected seed from wild-type plants showed pathogen colonization and did not germinate.

Finally, we determined whether *AtNPR1* lines exhibit resistance to *Erwinia chrysanthemi*, a bacterial pathogen that causes the foot rot disease of rice (Goto 1979). Ten days after infection, nontransformed seedlings showed typical symptoms of *E. chrysanthemi* infection, the roots becoming decayed and easily pulling off (Fig. 4, upper panels). Transgenic lines consistently performed better than controls, developed roots normally, and grew more vigorously (Fig. 4, lower panels). We further tested the effect of *E. chrysanthemi* infection on wild-type and transgenic lines by determining the length of inoculated roots relative to that of noninoculated roots for each line (in each case,

an arbitrary value of 1 was given to roots of noninfected seedlings). At 6 days after inoculation, the relative growth of infected roots from wild-type seedlings compared with noninfected roots was 0.27, whereas the relative growth for each transgenic line (inoculated versus noninoculated plants) was 0.80 for line 1, 0.70 for line 13, and 0.94 for line 24 (data are the means $\pm$ standard error of 60 plants). Together, disease resistance assays demonstrated that expression of *AtNPR1* in rice confers resistance to fungal and bacterial pathogens.

Priming for enhanced responses to fungal infection in *AtNPR1* rice plants.

The expression and pattern of fungal induction of the endogenous defense genes was examined in *AtNPR1* plants and com-

pared with that of wild-type plants. For this, total RNA was isolated from leaves of control and *AtNPR1* lines at different times after inoculation with *M. oryzae* spores. Total RNAs were then used for reverse-transcriptase polymerase chain reaction (RT-PCR) experiments using gene-specific primers for the *OsPR1b*, *probenazole-inducible 1* (*PBZ1*), *OsPR10*, and *PR5* (thaumatin-like protein [*TLP*]) genes. These genes have been identified as defense markers of the rice response to pathogen infection (Agrawal et al. 2001; Midoh and Iwata 1996; Velazhahan et al. 1998). None of these genes was expressed in the absence of the pathogen in either wild-type or transgenic plants (Fig. 5A). In fungus-infected *AtNPR1* plants, transcripts for all four rice defense genes accumulated earlier and at higher levels than in fungus-infected wild-type plants

Fig. 1. Expression of *AtNPR1* in rice plants. **A**, Northern blot analysis of transgenic rice plants (T0 generation). Total RNAs (10 μ g) were subjected to formaldehyde-containing agarose gel electrophoresis and hybridized with a 32 P-labeled cDNA probe. Lane WT, untransformed Senia control. Lower panels show ethidium bromide staining of RNA samples. **B**, Mean height of *AtNPR1* rice plants (T2 homozygous lines) compared with WT plants. Four independently generated *AtNPR1* lines (lines 1, 2, 13, and 24) and WT plants were grown in the greenhouse under control conditions for 3 weeks. Means were calculated from four independent transgenic lines with 48 plants for each line, and two repeats. The data represent mean and standard error ($P < 0.05$). **C**, Weight of seed produced by *AtNPR1* and WT rice plants. Seed were collected from transgenic and wild-type plants and pooled (48 plants per line); 200 seeds from each pool were weighted and the mean weight per seed was calculated. The data represent the mean weight and standard error ($P < 0.05$).

Fig. 2. Development of spontaneous lesions and expression of histochemical markers in the *AtNPR1* plants grown under growth-chamber conditions. **A**, Plants were grown in the greenhouse for 3 weeks and then transferred to the growth chamber. Lesion phenotype of *AtNPR1* plants at 4, 6, 8 and 10 days are shown. Leaves of wild-type (WT) plants grown in the growth chamber for 10 days did not develop lesions. **B**, Histochemical detection of superoxide in *AtNPR1* plants grown for 3 days in the growth chamber. Leaves were stained with nitro blue tetrazolium (NBT). Reduction of NBT to formazan yields a bluish precipitate in the presence of O_2^- . **C**, Detection of autofluorescent material in *AtNPR1* plants grown under growth-chamber conditions. Leaves were viewed by fluorescence (upper panels) or bright-field (lower panels) microscopy from the top side. Autofluorescent material accumulated in the cell walls in the leaves of transgenic lines (arrows) but not in leaves of WT plants.

Fig. 3. Resistance of rice plants expressing the *AtNPR1* gene to infection by the fungal pathogens **A** through **C**, *Magnaporthe oryzae* and **D**, *Fusarium verticillioides*. Similar results were observed with the four independent *AtNPR1* lines under study (lines 1, 2, 13, and 24). **A**, Resistance to infection by *M. oryzae*. Leaves from control untransformed Senia plants (WT) and *AtNPR1* rice lines were locally inoculated with *M. oryzae* spores. The concentration of inoculum is indicated on the left. Disease symptoms 3 days after inoculation of leaves are shown. Results shown are from one of four experiments, that produced similar results. **B**, Size of lesions (cm²) produced on the leaves of the control and *AtNPR1* rice lines 6 days after inoculation with *M. oryzae* spores. For inoculation, 20 μ l of a spore suspension at 1×10^6 spores/ml was used. The average lesion area was determined from spot infections (three inoculations per leaf, three leaves per plant, and three plants per line). Error bars represent standard deviation (SD). **C**, Counting of spores produced in *M. oryzae*-infected leaves at 6 days after inoculation. Values given represent the mean and standard error for three countings for each infected leaf and three leaves for each transgenic line. **D**, Resistance to infection by *F. verticillioides*. Seed were germinated for 24 h, inoculated with 50 μ l of fungal spores (10^7 spores/ml) (upper panels) or with sterile water (lower panels), and then allowed to continue germination. Picture was taken at 9 days after inoculation with fungal spores. Pictures shown are from one of four experiments that gave similar results. The percentage of seed that germinated under infection conditions relative to noninfected seeds was scored for each line at 6 days after inoculation with fungal spores (the histogram shows the means $\pm$ SD of four independent experiments).

(Fig. 5A). The four independently generated *AtNPR1* rice lines studied in this work (lines 1, 2, 13, and 24) showed faster and stronger activation of the *OsPR1b*, *PBZ1*, *TLP*, and *OsPR10* genes, supporting the idea that *AtNPR1* plants exhibit an enhanced capacity to activate the natural plant defense mechanisms, a phenomenon known as “priming” (Conrath et al. 2006).

Next, the response of the rice defense genes under study to treatment with SA was investigated. Treatment with SA strongly induces the expression of the *OsPR1b*, *TLP*, *OsPR10*, and *PBZ1* genes in both wild-type and transgenic rice plants (Fig. 5B). These results suggest that, under infection conditions, the expression of *AtNPR1* in rice primes for enhanced responses of endogenous genes which also show SA responsiveness in rice.

Finally, we investigated whether development of spontaneous lesions in transgenic plants grown under growth-chamber conditions was accompanied by the expression of defense-related genes in *AtNPR1* plants. For this, wild-type and transgenic lines were grown for 2 weeks in the greenhouse and transferred to the growth chamber for 3 days. By this time, expression of the *PR1b*, *PBZ1*, *TLP* (*PR5*), and *OsPR10* genes was found to be activated in leaves of *AtNPR1* plants but not in leaves of wild-type plants, which were maintained under the same growing conditions (Fig. 5C).

***AtNPR1* rice plants show altered expression of RYMV-responsive genes.**

In this work, we also examined the expression of rice genes whose expression is associated to the rice response to viral infections. Previous proteomic studies by Ventelon-Debout and

associates (2004) indicated that infection by RYMV results in alterations in the level of accumulation of a large number of rice proteins, including PR10A (accession number Q9LKJ9), the translation initiation factor 5A (*tIF5A*, accession number Q9ZSU2), and a salt-stress-induced protein (accession number P24120). PR10A is a member of the PR10 family of rice PR proteins which is different from that showing fungal responsiveness in rice plants (Fig. 5A). In this work, total RNA was extracted from locally inoculated (7 days postinoculation [dpi]), and mock-inoculated leaves of *AtNPR1* and wild-type plants and used for expression studies. In the absence of RYMV infection, no expression was observed for any of the rice genes under study (*tIF5A*, *salt-stress induced protein*, and *OsPR10A*) in either wild-type or transgenic plants (Fig. 6A). Upon infection of wild-type plants with RYMV, induction of *tIF5A* and *salt-stress-induced protein* gene expression, but not *OsPR10A* expression, occurred in wild-type plants. An opposite response to RYMV infection was observed in *AtNPR1* plants. In this case, *OsPR10A* expression but not *tIF5A* and *salt-stress-induced protein* expression was induced by RYMV infection. Thus, this study revealed that transgenic expression of *AtNPR1* results in altered expression of RYMV-responsive rice genes, at least of the *OsPR10A*, *tIF5A*, and *salt-stress-induced protein* rice genes. We also investigated the expression of the *tIF5A* and *OsPR10A* genes in two reference rice cultivars, Gigante and IR64 (highly resistant and highly susceptible to RYMV infection, respectively) (discussed below). This study revealed a similar response to RYMV infection in *AtNPR1* and IR64 plants (i.e., viral infection induced *OsPR10* but not *tIF5A*

Fig. 4. Resistance of *AtNPR1* plants to the bacterial foot rot *Erwinia chrysanthemi*. Disease resistance assays were carried out twice with three independent *AtNPR1* lines. Seed were germinated for 24 h, vacuum infiltrated with a bacterial culture (10^6 CFU), and then allowed to continue germination. Pictures were taken 10 days after bacterial infiltration. A detailed view of *AtNPR1* and wild-type (WT) roots is shown in the middle and right panels. This experiment is one of the three performed which gave similar results.

expression in both transgenic and IR64 plants at 7 dpi) whereas, in Gigante plants, RYMV infection induced *tIF5A* but not *OsPR10A* expression (as was also observed in virally infected wild-type plants) (results not shown).

Because of the close connection between expression of *RDR1* and antiviral defenses, we searched into the rice genome for the presence of *RDR1* sequences. A gene encoding a protein showing a high degree of identity to the *AtRdRP1* and *NtRDR1* genes was identified in the rice genome (accession

number NM00105486) (Dean et al. 2005). The protein encoded by this rice gene shares 63 and 64% identical amino acid residues with *AtRdRP1* and *NtRdRP1*, respectively. A phylogenetic analysis demonstrated that the gene identified in the rice genome clustered together with the *Arabidopsis* and tobacco *RDR1* genes (*AtRdRP1* and *NtRdRP1*), this gene being more distantly related to the *Arabidopsis* *RDR2* and *RDR6* genes (Fig. 6B). We named this rice gene *OsRDR1*. By using *OsRDR1*-specific PCR primers, we demonstrated that, like

Fig. 5. Expression of rice defense genes in *AtNPR1* and wild-type (WT) plants. Results for the *AtNPR1* line 13 are shown; similar results were obtained for the *AtNPR1* lines 1, 2, and 24. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using specific primers for the indicated genes: *OsPR1b*, *PBZ1*, *OsPR10*, and *TLP (PR5)*. **A**, RT-PCR analysis of *Magnaporthe oryzae*-infected (+) and noninfected (–) leaves from WT and *AtNPR1* plants. Each RNA was prepared from a pool from samples of leaves from eight plants at the three-leaf stage. Transcripts for the rice defense genes accumulated faster and at higher levels in fungus-infected *AtNPR1* plants than in fungus-infected WT plants. A low level of *OsPR10* transcript accumulation was consistently observed in control, noninfected WT plants (12 and 24 h). **B**, Effects of treatment with salicylic acid (2 mM) on the expression of rice defense genes. Control and transgenic plants were treated for 24 h. **C**, Expression of rice defense genes in leaves of transgenic and WT plants grown in growth chamber for 5 days.

NtRDR1 and *AtRDR1*, *OsRDR1* is also induced by SA treatment in rice plants (Fig. 6C).

Next, the effect of RYMV infection on *OsRDR1* gene expression was analyzed in wild-type and transgenic plants. In the absence of viral infection, *OsRDR1* expression is not detected in leaves of wild-type plants (Fig. 6D, 7, 10, and 14 dpi, upper panels). Upon infection of wild-type plants by RYMV, *OsRDR1* expression was not detected in either locally inoculated leaves or systemic leaves at 10 dpi, although a certain level of expression was observed in systemic leaves at 14 dpi (Fig. 6D). Contrary to this, *OsRDR1* gene expression occurred in both locally inoculated leaves (7 dpi) and systemic leaves (10 and 14 dpi) of RYMV-infected rice expressing *AtNPR1* (Fig. 6D). The RYMV-induced expression of *OsRDR1* increased with time, with higher levels of *OsRDR1* transcripts accumulating at 14 than at 10 dpi in leaves of *AtNPR1* plants. In other experiments, we confirmed that transcripts of the *AtNPR1* transgene accumulated at similar levels in leaves of *AtNPR1* plants, excluding the possibility of *OsRDR1*-mediated transgene silencing in transgenic plants (results not shown).

Enhanced susceptibility of *AtNPR1* rice plants against RYMV.

Knowing that the pattern of expression of RYMV-responsive rice genes is misregulated in *AtNPR1* plants, we investigated whether transgenic expression of *AtNPR1* in rice has an influence on the resistance against RYMV. Toward this end, T2 homozygous plants of five independent transgenic lines (lines 1, 2, 13, 24, and 27) and wild-type Senia plants were challenged with RYMV. Four rice cultivars, the two *indica* cvs. Gigante and IR64 (highly resistant and highly susceptible to RYMV, respectively) and the two *japonica* cvs. Azucena and Nipponbare (partially resistant and partially tolerant to RYMV, respectively) were used as reference cultivars in these assays (Albar et al. 1998; Ndjioudjop et al. 1999).

For challenge experiments, transgenic and control plants (70 plants per line) were mechanically inoculated with RYMV particles. Thirty plants of the same lines were mock inoculated with buffer and grown under the same experimental conditions. Development of disease symptoms and viral accumulation were monitored with time on both locally inoculated leaves (7 dpi) and newly developed leaves (systemic leaves, 10, 14, 21, and 28 dpi) (Figs. 7 and 8). At 7 dpi, symptoms characteristic of RYMV infection were already seen on the inoculated leaves of the highly susceptible cv. IR64 but not in the wild-type Senia or *AtNPR1* plants. However, at 10 to 14 dpi, all the transgenic rice lines developed symptoms of infection in systemic leaves. Thus, their leaves showed disperse chlorotic spots progressing to streaking lesions (Fig. 7A). By this time, disease symptoms were less evident in leaves of wild-type (Senia) plants. At 21 dpi, all transgenic plants exhibited pale, mottled leaves, disease symptoms being clearly more severe in transgenic plants than in wild-type plants (Fig. 7A). At 28 dpi, *AtNPR1* lines showed clear mottling and generalized yellowing, whereas wild-type plants appeared to be much healthier (Fig. 7B).

Scoring of symptom severity in wild-type and transgenic plants was performed using a 1-to-9 scale of the Standard Evaluation System for rice (SES), where 1 = no symptom and 9 = typical susceptible-type lesion (as indicated below). Symptom scale 5 was the typical response for the wild-type Senia and Nipponbare cultivars whereas symptom scale 7 was observed for all five *AtNPR1* rice plants assayed in this work (Fig. 7A, SES website).

Next, the level of virus accumulation was determined in locally inoculated leaves (7 dpi) as well as in systemic leaves (10, 14, and 21 dpi). Results are presented in Figure 8. At 7 dpi, the level of accumulation of RYMV in leaves of both transgenic and

Fig. 6. Expression of Rice yellow mottle virus (RYMV)-associated genes in *AtNPR1* and wild-type (WT) plants. **A**, Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of locally inoculated leaves (7 days postinoculation [dpi]) from wild-type (WT) and *AtNPR1* plants using specific primers for the *tif5A*, salt-stress-induced protein, and *PR10A* genes. **B**, Phylogenetic analysis of the relationship between the *OsRDR1* protein and the *Arabidopsis* and tobacco RNA-dependent RNA polymerase (RDR) proteins. **C**, Effect of treatment with salicylic acid (2 mM) on *OsRDR1* gene expression in WT rice. **D**, RT-PCR analysis of locally inoculated (7 dpi) and systemically infected leaves of RYMV-infected plants (10 and 14 dpi) using specific primers for the *OsRDR1* gene.

Fig. 7. Susceptibility of *AtNPR1* plants to Rice yellow mottle virus (RYMV). Five independent *AtNPR1* lines were assayed (lines 1, 2, 13, 24, and 27). Results for the *AtNPR1* lines 1, 2, and 13 are presented (similar results were obtained for the *AtNPR1* lines 24 and 27). **A**, Symptom severity in leaves of transgenic and wild-type (WT) plants inoculated with RYMV. Photographs were taken at 7 days postinoculation (dpi) (locally inoculated leaves) and at 10, 14, and 21 dpi (systemic leaves). Reference cultivars used in this study were Gigante (G, highly resistant), Azucena (A, partially resistant), Nipponbare (Np, partially tolerant), and IR64 (highly susceptible). Numbers at the bottom indicate the response of the different lines to RYMV infection according to the standard evaluation system for rice. Statistically significant differences between *AtNPR1* lines and WT plants are indicated by * ($P < 0.001$, analysis of variance with Tukey's highly significant difference; $\alpha = 0.05$). **B**, Phenotype of transgenic and WT plants inoculated with RYMV. The picture was taken at 28 dpi. Experiments were carried out three times. Similar results were observed in all the experiments.

wild-type plants was lower than in Azucena or Nipponbare plants (partially resistant and partially tolerant to RYMV, respectively). Consistently with the appearance of virus symptoms, the susceptible control cv. IR64 accumulated high levels of RYMV in both local and systemic leaves whereas the resistant cv. Gigante accumulated RYMV at very low levels. At 10 to 14 dpi, the level of virus accumulation in the systemic leaves of all the transgenic lines was significantly lower than in systemic leaves of wild-type plants. By this time, however, symptom severity was higher in systemic leaves of transgenic plants than in systemic leaves of wild-type plants (Fig. 7). Thus, there was no correlation between virus content and symptom expression in systemic leaves of transgenic plants when compared with wild-type plants. Virus content in systemic leaves of transgenic lines progressively increased from 10 to 14 and 21 dpi. At 21 dpi, the level of viral accumulation did not differ among the different transgenic and wild-type plants, although transgenic lines still developed more severe symptoms of infection than wild-type plants.

Together, and consistent with the observed misregulation of RYMV-responsive genes, these results revealed that constitutive expression of *AtNPR1* in transgenic rice led to an enhanced disease susceptibility to RYMV infection.

AtNPR1 negatively modulates expression of salt- and drought-associated rice genes.

We investigated whether transgenic expression of *AtNPR1* in rice has an effect on the expression of genes which are known to play a role in adaptation to drought and high salinity conditions and, if so, whether *AtNPR1* plants exhibit a phenotype of tolerance or sensitivity to high salt conditions. We selected specific rice genes whose expression was described to be upregulated in response to drought and high salinity, namely the *rab21*, a rice dehydrin (accession number AK109096), *salt* (*salt-stress-induced protein*, accession number AF001395), and *dip1* (*dehydration-stress inducible protein 1*, accession number AY587109) genes (Claes et al. 1990; Oh et al. 2005; Rabbani et al. 2003). The expression of these genes was analyzed by RT-PCR in roots of wild-type and

transgenic plants under salt stress conditions (150 mM NaCl). Under normal growth conditions, transgenic expression of *AtNPR1* in rice does not affect the expression of any of the genes under study (results not shown). In the case of the *rab21* gene, induction of its expression in response to salt treatment was delayed in transgenic plants compared with wild-type plants (Fig. 9). Moreover, *rab21* transcripts accumulated at lower levels in transgenic plants compared with wild-type plants. A delayed induction of *salt* gene expression was also observed in *AtNPR1* lines compared with wild-type plants. Finally, induction of *dip1* gene expression occurred at the same time after the onset of salt treatment (3 h) in transgenic and wild-type plants. In contrast to wild-type plants, accumulation of *dip1* transcripts progressively decreased with time (Fig. 9). Overall, this study revealed that expression of *AtNPR1* in rice causes a delay or reduces the intensity of expression of the salt-associated *rab21*, *salt*, and *dip1* rice genes.

AtNPR1 rice plants are hypersensitive to salt and drought stresses.

The tolerance of transgenic rice plants to drought and salt stresses was compared with that of control plants. Stress tolerance was evaluated based on the percentage of plants that survived after a period of drought or salt treatment. For this study, 2-week-old transgenic plants (lines 1, 2, 13, and 24; $n = 72$ plants per line) and wild-type plants (82 plants) were assayed. The tolerance of the transgenic plants to high-salt stress was examined by keeping the transgenic plants in NaCl at different concentrations (50, 100, and 200 mM NaCl) for 5 days and then growing them for 15 days with normal watering. All transgenic lines exhibited enhanced sensitivity to salt stress (Fig. 10; Table 1). The phenotypic difference between wild-type and transgenic plants was already observed after treatment with only 50 mM NaCl. At 2 weeks after treatment with 50 mM NaCl, none of the transgenic plants survived, whereas 96% of the wild-type plants survived.

Knowing that transgenic lines show a reduction in their size compared with wild-type plants, there was the possibility that a reduction in the plant size might have an effect on tolerance

Fig. 8. Virus accumulation in leaves of transgenic and wild-type (WT) plants inoculated with *Rice yellow mottle virus* (RYMV). WT (white bars), transgenic lines (black bars) and reference cultivars (dashed bars) Gigante (G), Azucena (A), Nipponbare (Np), and IR64. Leaves from nine plants for each line were pooled and the virus content determined by enzyme-linked immunosorbent assay using an antibody generated against the coat protein as described (N'Guessan et al. 2000). Accumulation of RYMV in **A**, locally inoculated leaves at 7 days postinoculation (dpi) and **B** through **D**, systemically infected leaves at 10, 14, and 21 dpi, respectively, from WT, *AtNPR1* lines, and the control cvs. Gigante (G), IR64, Azucena (A), and Nipponbare (Np) is presented. Statistically significant differences between *AtNPR1* lines and WT plants are indicated by * ($P < 0.001$, analysis of variance with Tukey's highly significant difference; $\alpha = 0.05$).

to salt stress. During the course of this work, salt tolerance assays were also carried out with 2-day-germinated seedlings from wild-type and transgenic lines. Seedlings were also subjected to salt treatment for 5 days. Results obtained in these experiments also revealed a higher susceptibility to salt treatment in *AtNPR1* lines compared with wild-type plants (results not shown).

To determine the tolerance of transgenic rice to drought, we grew transgenic and wild-type plants without watering for 9 days and then supplying water again for 2 weeks. All transgenic plants, but not wild-type plants, showed wilting and drought-induced rolling of young leaves (Fig. 10). Whereas 90% of the wild-type plants survived 9 days without water, the survival rate for transgenic lines ranged from 46 to 61%, depending on the line (Table 2). From these results, it is concluded that expression of *AtNPR1* in transgenic rice causes hypersensitivity to both salt and drought stresses.

DISCUSSION

The development of genetically engineered disease-resistant plants comes as a result of efforts to understand the molecular mechanisms involved in the plant defense response against pathogens. A better understanding of the function of regulatory components of the plant defense response, and the target genes and traits that they regulate, is of great importance not only for basic research in plant biology but also for crop protection. Presumably, the use of regulatory genes of the plant defense mechanisms might allow the activation of the expression of multiple defense genes and, accordingly, better levels of protection are expected compared with strategies based on the use of a single gene or a combination of individual genes encoding antimicrobial proteins.

Compelling evidence exists that *AtNPR1*, when introduced into a plant, confers resistance to fungal and bacterial diseases,

Fig. 9. Expression of salt- and drought-associated rice genes *rab21*, *saltT*, and *dip1* in wild-type (WT) and transgenic plants. T2 homozygous *AtNPR1* rice lines and WT plants were grown in Murashige-Skoog medium for 7 days and then exposed to water (control) or 150 mM NaCl for 1, 3, 6, 9, or 24 h. **A**, Representative results obtained by reverse-transcriptase polymerase chain reaction analysis of the *rab21*, *saltT*, and *dip1* genes in *AtNPR1* line 1 are shown. **B**, Relative changes of *rab21*, *saltT* and *dip1* transcript levels plotted to the highest value after normalization with *Actin 1*. Values represent the means $\pm$ standard deviation of three independent experiments in *AtNPR1* line 1. Similar results were observed for lines 2 and 13.

Fig. 10. Sensitivity of *AtNPR1* plants to salt and drought stress. All four independently generated transgenic lines assayed in this work (lines 1, 2, 13, and 24 and T2 homozygous lines) showed hypersensitivity to salt and drought stress. **A** through **C**, Phenotype of plants during salt stress conditions. Plants were grown in the greenhouse for 2 weeks, treated with NaCl (50, 100, and 200 mM) for 5 days, and then irrigated with water for 2 weeks. **D**, Drought stress; water was withheld for nine days and then plants were supplied with water again for 2 weeks. The pictures were taken at 15 days after salt or drought treatment. Assays to determine tolerance to salt and drought stress were carried out four times and at different times of the year. In each experiment, 84 wild-type (WT) plants and 73 plants per transgenic line were used. Similar results were observed in all the assays.

which led to the proposal of *AtNPR1* as a promising candidate gene for engineering broad-spectrum disease resistance in transgenic plants. In this work, the *Arabidopsis AtNPR1* gene was introduced into rice plants. Both positive and negative effects caused by *AtNPR1* expression in rice were found. Thus, the expression of *AtNPR1* in rice has a negative effect on plant growth and productivity. Moreover, *AtNPR1* plants develop spontaneous lesions when growing under suboptimal conditions (growth chamber). An important difference between greenhouse and growth-chamber conditions is the total light intensity (5,000 to 8,500 lux in the growth chamber and 12,000 to 20,000 lux in the greenhouse). In this respect, lesion formation in Taipei 309 rice plants expressing *AtNPR1* was already reported by Fitzgerald and associates (2004). However, whereas, in *AtNPR1* Senia plants, the lesions appeared 2 to 3 days after transferring the plants to the growth chamber (present work), in *AtNPR1* Taipei 309 plants, the lesions were apparent only after 2 to 3 weeks under growth-chamber conditions (Fitzgerald et al. 2004).

Expression of the *AtNPR1* gene in rice confers protection against fungal (*M. oryzae* and *F. verticillioides*) and bacterial (*E. chrysanthemi*) pathogens, further supporting a role for the *AtNPR1* gene in resistance to these types of pathogens in rice. The fungus *M. oryzae* was chosen for this study because this fungus causes rice blast, the most important fungal disease of cultivated rice (Talbot 2003). Breeding of durable resistance to *M. oryzae* is a difficult problem not only because of the high degree of pathogenic variability of this fungus but also because of the large number of fungal races encountered in the field population. The fungus *F. verticillioides*, formerly known as *F. moniliforme* Sheldon, is also an important fungal pathogen of rice and the causal agent of the bakanae disease. *F. verticillioides* causes a decrease not only in quantity but also in quality of rice by producing toxins, namely fumonisins, that affect

Table 1. Effect of salt treatment on *AtNPR1* rice plants^a

Lines	No. of plants	Survival no. (%) ^b	
		7 days	15 days
Wild type	84	84 (100)	81 (96)
<i>AtNPR1-1</i>	72	6 (9)*	0
<i>AtNPR1-2</i>	72	8 (11)*	0
<i>AtNPR1-13</i>	72	10 (14)*	0
<i>AtNPR1-24</i>	72	8 (11)*	0

^a Two-week old plants were treated with 50 mM NaCl for 5 days and then irrigated with water. The number of plants used from wild-type and transgenic (lines 1, 2, 13, and 24) is shown.

^b Number of plants that survived salt treatment after a period of recovery of 7 or 15 days. Data represent means $\pm$ standard deviation of four independent experiments. Numbers in parenthesis indicate the percentage of surviving plants; * indicates that statistically significant differences between *AtNPR1* and wild-type lines were observed (χ^2 test, $P < 0.001$).

Table 2. Survival of *AtNPR1* plants following drought treatment^a

Lines	No. of plants	Survival no. (%) ^b
Wild type	84	65 (90)
<i>AtNPR1-1</i>	72	38 (53)*
<i>AtNPR1-2</i>	72	45 (63)*
<i>AtNPR1-13</i>	72	33 (46)*
<i>AtNPR1-24</i>	72	44 (61)*

^a Water was completely withheld from 2-week-old plants for 9 days and then watering was resumed for 15 days. The number of plants used in this study, both wild-type and transgenic (lines 1, 2, 13, and 24) plants, is shown.

^b Number of plants that survived and survival rate (%) for each line after 15 days of rewatering. Data represent means $\pm$ standard deviation of four independent experiments. Numbers in parenthesis indicate the percentage of surviving plants; * indicates that statistically significant differences between *AtNPR1* and wild-type lines were observed (χ^2 test, $P < 0.005$).

human and animal health (Nelson et al. 1993). Finally, the gram-negative bacterium *E. chrysanthemi*, the causal agent of the foot rot disease of rice, represents another major constrain for rice production (Goto 1979). Results here presented show that *AtNPR1* confers resistance against foliar pathogens as well as against seed and root pathogens, illustrating the important role that *AtNPR1* plays in the plant defense response even early in plant development. On the other hand, studies carried out in *Arabidopsis* suggested that the plant defense responses may be tailored to the attacking pathogen, with SA-dependent defenses acting against biotrophs, and jasmonic (JA)- and ethylene (ET)-dependent responses acting against necrotrophs (Glazebrook 2005). In particular, *AtNPR1* has been implicated in resistance against biotrophic pathogens that requires SA signaling. The two fungal pathogens used in this study, *M. oryzae* and *F. verticillioides*, have been described as hemibiotrophic and necrotrophic fungi, respectively, whereas *Erwinia* spp. are bacterial necrotrophs. To our knowledge, no information is currently available about requirements for a specific signaling pathway, such as the SA-, JA-, or ET-dependent signaling pathway, during the rice response to these particular fungal and bacterial pathogens.

By examining the expression of marker genes of the endogenous defense response, we determined that *AtNPR1* plants are in a primed state, these plants exhibiting a faster and stronger activation of rice defense genes during pathogen infection compared with wild-type plants. Priming of defense-related genes is then consistent with the disease resistance phenotype observed in these plants. Here, it is worthwhile to mention that, although molecular information is available on the induced priming phenomenon in the dicotyledoneae (Conrath et al. 2006), this information is largely missing for monocotyledoneae, including the most important agronomic cereals. In this respect, our group recently reported a sucrose-mediated priming of defense responses in rice (Gómez-Ariza et al. 2007).

On the other hand, it is well known that the levels of SA dramatically increase in response to pathogen attack and during SAR in *Arabidopsis* and tobacco plants (Dempsey et al. 1999). In rice, however, the endogenous levels of SA are very high and its level does not change significantly after infection. The important question, then, is whether SA has a central role in induced defense responses in rice. Recent studies have shown that the signaling pathways downstream of SA are evolutionally divergent between rice and *Arabidopsis* (Shimono et al. 2007). When the SA responsiveness of the rice genes showing enhanced responses during pathogen infection in *AtNPR1* rice plants (*PR1b*, *TLP*, *OsPRI0*, and *PBZ1*) was examined, we found that these genes were strongly activated by treatment with SA. Then, the *AtNPR1*-mediated priming of defense gene expression in *AtNPR1* rice plants correlated well with the observed SA responsiveness of these genes. Future research on the *AtNPR1*-mediated priming mechanisms will allow a more comprehensive view of this phenomenon in rice plants.

Results presented here revealed novel functions for *AtNPR1* in controlling the plant response to viral pathogens, particularly to RYMV. Since its early description in 1974 (Bakker 1974), RYMV has become a model virus in rice. RYMV is a typical member of the genus *Sobemovirus*. The RYMV genome is a single-stranded messenger-sense RNA (ssRNA+) (Fargette et al. 2004). Although partial resistance is widely distributed in *O. sativa* cultivars, only some rare cultivars for the cultivated *O. sativa* and *O. glaberrima* species exhibit high resistance to RYMV (Ndjiondjop et al. 1999; Thottappilly and Rossel 1993). However, a recessive rice-resistant gene against RYMV encoding the eukaryotic translation initiation factor 4G (eIF4G) has been recently described (Albar et al. 2006). Results presented here show that *AtNPR1* interferes with the normal

expression of rice genes which have been associated with the rice response to RYMV, the *tIF5A*, *salt-stress-induced protein*, *PR10A*, and *OsRDR1* genes. The observed *AtNPR1*-mediated misregulation on the expression of RYMV-responsive rice genes has important consequences for the rice plant, the *AtNPR1* rice plants exhibiting enhanced susceptibility to RYMV infection. An intriguing aspect of this study came from the observation that, during the initial phase of systemic infection of *AtNPR1* plants by RYMV, there was no correlation between virus content and disease severity. By measuring the accumulation of RYMV in systemically infected leaves (10 and 14 dpi), we observed that RYMV-infected *AtNPR1* plants showed a lower level of accumulation of RYMV particles compared with RYMV-infected wild-type plants. Systemically infected leaves of transgenic plants, however, exhibited more severe disease symptoms than systemically infected leaves of wild-type plants. One possible explanation for the reduced level of viral accumulation in leaves of transgenic plants compared with wild-type plants is that the virus spreads to a lesser extent in the vascular tissue from the initial infection site during the early stages of systemic infection. It is tempting to speculate that the observed differential reaction to RYMV infection between transgenic and wild-type plants might be caused by the interference of *AtNPR1* with host factors which are usually positive regulators of the response against viral infection. An alternative explanation for the discrepancy observed between viral content and symptom intensity in *AtNPR1* plants could be that *AtNPR1* overexpression might cause an overreaction in the defense response to viral infection, leading to more severe disease symptoms.

During the course of this work, we identified a rice *RDR1* gene, the *OsRDR1* gene, showing responsiveness to SA. *RDR1* proteins are known to mediate RNA silencing pathways regulating defense against viruses (Baulcombe 2004). Leaves of *AtNPR1* lines were characterized by strikingly higher levels of *OsRDR1* expression compared with leaf tissues of wild-type plants. Theoretically, an increase in *OsRDR1* expression might result in a reduction of the accumulation of viral RNAs through the activation of RNA-silencing pathways and degradation of viral RNAs and, thereby, lowering the amount of viral particles in leaves of transgenic plants. Then, this mechanism might explain the lower level of virus particles observed in transgenic plants compared with wild-type plants at the early stages of the infection process. A similar function promoting turnover of viral RNAs has been postulated for the orthologous *Arabidopsis* (*AtRdRPI*) and tobacco (*NtRdRPI* and *NbRdRPI*) genes, for which induction by treatment with SA and viral infection has been also described (Yu et al. 2003). *AtNPR1* rice plants may be useful to further explore whether *AtNPR1* interferes with RNA silencing mechanisms in rice plants during RYMV infection. It will also be interesting to determine the effect of *AtNPR1* on the response of rice plants to viruses other than RYMV.

Mechanisms conferring resistance to fungi and bacteria are usually considered to function independently from those involved in resistance to viruses. Results presented here, however, support the idea that some cross-talk exists among these pathways, *AtNPR1* functioning by priming the expression of rice defense genes involved in resistance against fungal pathogens while altering the normal expression of virally associated rice genes. In agreement with this, constitutive expression of *AtNPR1* in rice confers resistance to fungal and bacterial pathogens while exerting a profound, negative effect on viral resistance in rice.

Until now, *AtNPR1* has not been functionally characterized in relation to abiotic stresses, particularly to salt and drought stress. However, *AtNPR1* appears to be required for ozone-

induced ethylene production and cell death (Rao et al. 2002). We found that *AtNPR1* negatively regulates the expression of genes functioning in salt- and drought-stress responses in rice, namely the *rab21*, *saltT*, and *dip1* rice genes. The observed negative regulation of salt or drought marker genes is accompanied by hypersensitivity to salt and drought stresses in *AtNPR1* plants. Taking into account the fact that signaling pathways involved in plant responses to salt and drought stress are largely overlapping (Seki et al. 2003; Zhu 2002), it is not surprising to find that *AtNPR1* rice plants are more sensitive to the two types of stress. Interestingly, it was reported that RYMV infection regulates the expression of genes for which a function has been traditionally associated with the plant response to salt stress, including the *lF5A*, *PR10A*, and *salt-associated protein* genes studied in this work (Ventelon-Debout et al. 2004). This might explain why *AtNPR1* rice plants are negatively affected in their response to salt and drought stress as well as to the stress imposed by viral infection. Finally, all independent transgenic lines used in the present study exhibited the same phenotype (reduced size and productivity and development of spontaneous lesions), behavior under biotic and abiotic stress conditions, and transgene-mediated alterations in host gene expression excluding the possibility of an insertional effect caused by transgene integration into the rice genome.

To conclude, this study allowed us to correlate the transcript levels of endogenous genes associated with different types of stress, both biotic and abiotic, with the observed phenotype of *AtNPR1* plants under these particular stress conditions. Collectively, the results presented here demonstrate the complexity of the roles played by *AtNPR1* in plant defense responses while illustrating the antagonistic relationships that occur between the various signaling pathways controlling the plant response to biotic and abiotic stresses. In other studies, a rice mitogen-activated protein kinase that negatively modulates resistance to fungal and bacterial pathogens and positively regulates abiotic stress tolerance was reported (Xiong and Yang 2003). One function of *AtNPR1* might be to provide a fine-tuning mechanism of the plant defense response and to coordinate responses associated with each type of stress. The possibility that *AtNPR1* regulates defense gene expression through the concerted action of positive and negative regulators associated with each specific type of stress defense response should be considered. Negative effects of *AtNPR1* on defense gene expression might result from suppression of positive regulators of abiotic stress responses and antiviral defenses (i.e., by sequestering positive regulators of the type of stress). Taking into account that transgenic expression of *AtNPR1* in rice is associated with a penalty under situations of viral infection and abiotic stress conditions, the practical question is whether the resistance conferred by transgenic expression of *AtNPR1* in rice could be used to improve resistance to fungal and bacterial pathogens under field conditions. The results described in this study point to the need to further dissect the pathways in which *AtNPR1* is involved in disease resistance and tolerance to abiotic stresses in plants.

MATERIALS AND METHODS

Plant material and growth conditions.

Rice plants (*O. sativa* L. cv. Senia) were grown in the greenhouse at 26 to 28°C with a 18- and 6-h (day and night, respectively) cycle. When needed, plants were transferred to growth chambers (5,000 to 8,500 lux) under the same diurnal cycle. For rice transformation, the *Agrobacterium tumefaciens* EAH105 was used to infect embryogenic callus derived from mature embryos according to Pons and associates (2000).

Construction of the plant expression vector and rice transformation.

The *Arabidopsis NPR1* cDNA sequence (kindly provided by X. Dong, Duke University, Durham, NC, U.S.A.) was amplified from the pKEX4tr plasmid (Cao et al. 1997) with primers 5'-GCGGATCCACACGATGGACACCACCATTTGA-3' and 5'-GCGGATCCCACTAAGAGGCAAGAGTC-3' (underlined letters indicate the *Bam*HI restriction site which was introduced at the 5' and 3' ends of the DNA for the subsequent cloning steps). The PCR-amplified *AtNPR1* DNA was cloned into the pGEM-T plasmid. On the other hand, the ubiquitin promoter and nos terminator DNA sequences were amplified with primers UBI5', 5'-GCGAGCTCCTGCAGTGCAGCGTGACCCGGTCGTGC-3' and NOS3', 5'-gcgagctccgatctagtaacatagat-3' containing the *Sac*I cloning site and cloned into the pGEM-T plasmid to obtain the *pGEMT:ubi:nos* plasmid. The *AtNPR1* cDNA fragment was then inserted into the *Bam*HI site of the *pGEMT:ubi:nos* plasmid under the control of the maize ubiquitin 1 promoter (*ubi1*) and the *nos* terminator. Finally, the entire DNA fragment containing the 35S*CaMV* promoter, *AtNPR1* cDNA, and terminator was cloned into the *Sac*I-digested pCAMBIA1300 vector to obtain the *pCAMBIA-ubi1::AtNPR1::nos* construct. The nucleotide sequence of the plant expression vector was verified by nucleotide sequencing. The parent pCAMBIA 1301 vector already contains the hygromycin phospho transferase (*hptII*) gene encoding hygromycin resistance in the T-DNA region.

RNA isolation and Northern blots.

Total RNA was isolated from leaves collected from soil-grown plants at the two to three-leaf stage using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.). RNAs were subjected to formaldehyde-containing agarose gel electrophoresis and transferred to nylon membranes (Hybond-N, Amersham, Chalfont, Buckinghamshire, U.K.). The DNA fragment coding for the *AtNPR1* gene was used as probe. Hybridizations were conducted at 42°C with the addition of 40% formamide in the hybridization solution.

RT-PCR analysis.

For RT-PCR of the rice defense genes, total RNA was extracted from leaves of rice plants at the three- to four-leaf stage at different times after fungal inoculation (3, 6, 12, and 24 h after inoculation with fungal spores). For each time-point, leaves were collected from eight individual plants. The first cDNA was synthesized with DNase-treated total RNA (1 µg) with *Moloney murine leukemia virus* (M-MLV) reverse transcriptase (Roche, Mannheim, Germany). Aliquots of the resulting RT reaction product were used as template for RT-PCR analysis. Primers used for amplification reactions were for *OsPR-1b* (forward, 5'-CTTGGCGAGAACCTCTTCTG-3'; reverse, 5'-GCCGGCTTATAGTTGCATGT-3'), *TLP (PR5)* (forward, 5'-CAGTACTGCTGCACCGGCTC-3'; reverse, 5'-ACATCGATCAGATGCCAGCTAA-3'), *PBZ1* (forward, 5'-CGGGCACCATCTACACC-3'; reverse, 5'-CCTCGATCATCTTGAGCATGC-3'), *OsPR10* (forward, 5'-TCAGGCAGTTCAACTTCACCTC-3'; reverse, 5'-CTTAGCCTTGGTGATCTCGTCC-3'), *saltT* (forward, 5'-GGAAATTTAAGCGCACCA-3'; reverse, 5'-GTCTTGCACTGGAATGCTGA-3'), *rab21* (forward, 5'-GGAATCCATTCACTGCCAAT-3'; reverse, 5'-TTGATCCCTTCTTCCTCCT-3'), *dip1* (forward, 5'-GGCTTTAGCTGAATCCATCG-3'; reverse, 5'-CTCCTTGAGCCCTTCTTCT-3'), *lF5A* (forward, 5'-GAGGAGCAACATTTTCGAGTC-3'; reverse, 5'-ACATCACAGTTGTGGGACGA-3'), *salt-stress-induced protein* (forward, 5'-CACCCAGAAGCTGTTAGGC-3'; reverse, 5'-GTCTTGCACTGGATGCTGA-3'), *OsPR10A* (forward, 5'-GGTGTGGGAAG

CACATACAA-3'; reverse, 5'-AGTAGCCATCCACGATGTC C-3'), and *OsRDR1* (forward, 5'-GCTACGTCCAACCATCA CCT-3'; reverse, 5'-TCATGTCCTGAAGCAGAACG-3'). The control of the rice *Actin 1* gene was performed using the forward primer 5'-CCTCTTCCAGCCTTCCTTCATA-3'; reverse 5'-GCAATGCCAGGGAACATAGTG-3'. Three independent RT-PCR reactions for the three independent *AtNPR1* lines were carried out. RT-PCR products were quantified using a phosphorimager and the Quantity One software (Bio Rad, Munchen, Germany) and normalized according to *Actin1*.

Phylogenetic analysis.

The phylogenetic tree was obtained by the Neighbor-joining method (Saitou and Nei 1987). The tree was displayed with MEGA 3.1 program (Kumar et al. 2004) showing bootstrap values (from 1,000 replicates) higher than 50%.

Microscopic analyses.

Microscopic analysis of leaves from transgenic and wild-type plants by bright-field and fluorescence microscopy was carried out as previously described (Dietrich et al. 1994). Cell autofluorescence was viewed by fluorescence microscopy (excitation filter 365, dichroic mirror 395 nm, barrier filter 420 nm) (Zeiss Axiophot microscope; Carl Zeiss, Thornwood, NY, U.S.A.). For histochemical detection of superoxide O_2^- , leaf sections of approximately 2 cm in length were excised from either transgenic or wild-type plants and stained as described by Fitzgerald and associates (2004).

Disease resistance assays with fungal and bacterial pathogens.

Resistance of *NPR1* rice plants to infection by the rice blast fungus *M. oryzae* (PR9 isolate, CIRAD collection, Montpellier, France) was assayed as previously described (Coca et al. 2004). The rice cv. Senia used for transformation in this work is highly susceptible to infection by the *M. oryzae* PR9 isolate (Coca et al. 2004). Blast resistance was also measured by counting the number of spores from wild-type and transgenic lines and by estimating the leaf area covered with lesions (Coca et al. 2006). The *F. verticillioides* isolate used in this work was collected from rice plants in Spain and supplied by the Facility of Plant Protection from the Generalitat de Catalunya. Evaluation of resistance to *F. verticillioides* and to *E. chrysanthemi* A3937 was carried out as described by Gómez-Ariza and associates (2007). For each line, germination was scored at day 6 after inoculation with *F. verticillioides* spores relative to that of noninoculated plants. Root length of *E. chrysanthemi*-infected seedlings from wild-type and transgenic plants was determined and compared with that of noninfected seedlings using the Image J software. Infections were carried out three times (60 plants per line).

Plant inoculation with RYMV and enzyme-linked immunosorbent assay.

Transgenic and wild-type plants (70 plants per line) were grown under controlled conditions in a biosafety level 2 greenhouse (IRD, Montpellier, France). Fifteen-day-old homozygous T2 plants were inoculated with RYMV particles diluted in phosphate buffer (20 mM KH_2PO_4 and K_2HPO_4 , pH 7.2; 50 µg/ml) by finger rubbing the leaves in the presence of Carborundum (Kouassi et al. 2006). Thirty plants of the same lines were mock inoculated with RYMV and grown under the same experimental conditions. Resistance to RYMV infection of *AtNPR1* plants was compared with that of two *O. sativa indica* cultivars, IR64 and Gigante, and two *O. sativa japonica* cultivars, Azucena and Nipponbare. Contrary to what is found in

most of the *indica* cultivars which are very susceptible to RYMV, the Gigante *indica* cultivar shows a high degree of resistance to RYMV (Albar et al. 2006). On the other hand, the *indica* cv. IR64 is highly susceptible to RYMV. The two *japonica* cultivars used in this work, Azucena and Nipponbare, are partially resistant and partially tolerant, respectively, to RYMV.

Symptom severity at 28 dpi was evaluated following the 1-to-9 scale developed by the International Rice Research Institute (Los Banos, Philippines) SES. Typical responses of rice plants to RYMV are as follows: symptom scale 1 = no symptoms observed, a response typical of the highly resistant cv. Gigante; symptom scales 2 and 3 = sparse chlorotic spots or streaking on green leaves, seen in plants such as cv. Azucena; symptom scale 5 = plant exhibiting pale green leaves with mottling, seen in plants such as Senia and Nipponbare; and symptom scales 7 to 9 = yellow to orange leaves, typical of the highly susceptible cv. IR64. Analysis of variance and the Tukey's multiple comparisons test were used to compare susceptibility of rice plants to RYMV using XLSTAT software (Addinsoft, Brooklyn, NY, U.S.A.).

The level of virus accumulation was determined at different days postinoculation measured in locally inoculated leaves (7 dpi) and systemically infected leaves (10, 14, and 21 dpi) by enzyme-linked immunosorbent assay analyses using an antibody against RYMV coat protein (N'Guessan et al. 2000). Leaves from 10 inoculated plants for each line were pooled and the virus content determined. Extracts were prepared using 0.05 M Na phosphate, pH 7.5, and 0.15 M NaCl containing 0.1% Tween-20 as the extraction buffer.

Abiotic and chemical treatment of plants.

Wild-type (84 plants) and transgenic lines (four independent T2 homozygous lines and 72 plants per line) were grown under greenhouse conditions for 2 weeks. For salt-stress treatment, plants were transferred to flat trays containing NaCl for up to 5 days. Plants were then irrigated with water for 15 days. Each plant was grown in the same amount of soil and watered with the same volume of water. Salt treatments were also performed with in-vitro-grown rice seedlings. For this, seed from wild-type and transgenic lines (80 plants per line) were pregerminated in Murashige-Skoog medium containing gelrite at 0.85 g/liter and then treated with either NaCl (50, 100, 150, or 200 mM), mannitol (300 mM), or sterile water for 5 days.

Drought stress was induced by withholding water for up to 9 days. Plants were then irrigated normally for 15 days. Drought and salt tolerance were evaluated by determining the percentage of surviving plants after the period of recovery. Experiments to determine tolerance to salt and drought stresses were repeated four times and at different times of the year. Statistical analysis of data obtained in these experiments was performed using the χ^2 test at a 5% confidence level.

For treatment with SA, 2-week-old plants were sprayed with a solution of 2 mM SA. Control plants were treated with water. Plants were collected at 24 h after the onset of treatment.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

International Rice Research Institute Standar Evaluation System for Rice (SES): www.knowledgebank.irri.org/ses/SES.htm
 Invitrogen website: www.invitrogen.com
 National Center for Biotechnology Information: www.ncbi.nlm.nih.gov
 National Institute of Health Image J software: rsb.info.nih.gov/ij