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Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility†

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Summary

The key regulator of salicylic acid (SA)-mediated resistance, NPR1, is functionally conserved in diverse plant species, including rice (*Oryza sativa* L.). Investigation in depth is needed to provide an understanding of *NPR1*-mediated resistance and a practical strategy for the improvement of disease resistance in the model crop rice. The rice genome contains five *NPR1*-like genes. In our study, three rice homologous genes, *OsNPR1/NH1*, *OsNPR2/NH2* and *OsNPR3*, were found to be induced by rice bacterial blight *Xanthomonas oryzae* pv. *oryzae* and rice blast *Magnaporthe grisea*, and the defence molecules benzothiadiazole, methyl jasmonate and ethylene. We confirmed that *OsNPR1* is the rice orthologue by complementing the *Arabidopsis npr1* mutant. Over-expression of *OsNPR1* conferred disease resistance to bacterial blight, but also enhanced herbivore susceptibility in transgenic plants. The OsNPR1-green fluorescent protein (GFP) fusion protein was localized in the cytoplasm and moved into the nucleus after redox change. Mutations in its conserved cysteine residues led to the constitutive localization of OsNPR1(2CA)-GFP in the nucleus and also abolished herbivore hypersensitivity in transgenic rice. Different subcellular localizations of OsNPR1 antagonistically regulated SA- and jasmonic acid (JA)-responsive genes, but not SA and JA levels, indicating that OsNPR1 might mediate antagonistic cross-talk between the SA- and JA-dependent pathways in rice. This study demonstrates that rice has evolved an SA-mediated systemic acquired resistance similar to that in Arabidopsis, and also provides a practical approach for the improvement of disease resistance without the penalty of decreased herbivore resistance in rice.

Introduction

Plants have evolved a set of defence mechanisms against pathogens, which include pre-existing physical and chemical barriers, and induced defence responses to pathogen attack. The latter occur at the site of infection, as well as in uninfected distant tissues. The resistance spreading throughout the whole plant is termed systemic acquired resistance (SAR) or induced systemic resistance (ISR). SAR is triggered by local infection with pathogens, is characterized by pathogenesis-related (*PR*) gene expression in distal tissues, and is commonly recognized as efficient broad-spectrum disease resistance (Pieterse *et al*., 1996, 1998; Ryals *et al*., 1996; Sticher *et al*., 1997). Extensive studies in dicotyledonous plants, including *Arabidopsis* and tobacco, have shown that salicylic acid (SA) is an essential signal molecule for SAR. SA levels increase in both infected and distal leaves in response to pathogen attack, and SA is required for the induction of a set of *PR*

genes (Klessig and Malamy, 1994; Summermatter *et al*., 1995). By contrast, ISR, characterized by *PDF1.2* gene expression in distal tissues, requires the signal molecules jasmonic acid (JA) and/or ethylene (ET), and confers resistance to insects and some necrotrophic pathogens (Kessler and Baldwin, 2002; Durrant and Dong, 2004; Gfeller *et al*., 2006). Although a few reports have shown examples of synergistic interactions between JA and SA signalling, these two hormones interact in an antagonistic manner, with, in general, an epistatic effect of SA signalling on JA signalling.

Extensive studies have shown that the *NPR1* gene (also known as *NIM1* and *SAI1*) functions as the key regulator of SA-mediated SAR in the model plant *Arabidopsis*. The mutants of the gene, *npr1/nim1/sai1*, have lost the expression of SA-induced *PR* genes, SAR and some resistance (*R*) gene-mediated resistance (Cao *et al*., 1994; Delaney *et al*., 1995; Glazebrook *et al*., 1996; Shah *et al*., 1997). Over-expression of *NPR1* enhances resistance to diverse pathogens, including bacteria and fungi, in a dosage-dependent manner in *Arabidopsis* (Cao *et al*., 1998; Friedrich *et al*., 2001). The NPR1 protein contains a bipartite nuclear localization sequence and two protein–protein interaction domains, an ankyrin repeat domain and a BTB/POZ (Broad complex, Tramtrack and Bric-a-brac/Pox virus and Zinc finger) domain (Cao *et al*., 1997; Ryals *et al*., 1997). It has been documented that NPR1 differentially interacts with the TGA family members of basic domain/Leu zipper (bZIP) transcription factors to induce the expression of downstream *PR* genes (Zhang *et al*., 1999; Despres *et al*., 2000; Zhou *et al*., 2000; Kim and Delaney, 2002; Johnson *et al*., 2003). A recent study has shown that NPR1 also coordinately induces secretion-related genes required for PR protein secretion during SAR (Wang *et al*., 2005). The *Arabidopsis* genome contains five *NPR1* homologous genes; *NPR3* and *NPR4* also function in plant disease resistance, and NPR3 and NPR4 negatively regulate *PR* gene expression and pathogen resistance through their association with TGA2 and its paralogues (Liu *et al*., 2005; Zhang *et al*., 2006).

NPR1 is constitutively expressed in *Arabidopsis*, and can be further induced by SA or 2,6-dichloroisonicotinic acid (INA, a type of SA analogue) treatment (Cao *et al*., 1997) or by pathogen infection (Ryals *et al*., 1997). However, constitutive expression of *NPR1* in the absence of an inducer does not lead to the expression of *PR* genes, indicating that activation of the NPR1 protein must also occur on SAR induction (Cao *et al*., 1997). Studies have demonstrated that NPR1 accumulates in the cytoplasm in an oligomeric form. During SAR induced by SA and pathogen, it is converted into a monomer and moves into the nucleus to induce *PR* genes, a process essential for its function (Kinkema *et al*., 2000; Mou *et al*., 2003). It has been shown that the two conserved cysteine residues (C82 and C216) play an essential role in NPR1 oligomer formation. Mutations in these residues cause constitutive monomerization, localization of NPR1 in the nucleus and defence gene expression (Mou *et al*., 2003). NPR1 also mediates cross-talk between the SA signalling pathway and the JA signalling pathway, and the antagonistic effect of SA on JA signalling requires NPR1 (Spoel *et al*., 2003). The *npr1* mutant exhibits enhanced resistance to cabbage looper and Egyptian cotton worm (Cui *et al*., 2002; Stotz *et al*., 2002). However, nuclear localization of NPR1 is not required for the suppression of JA signalling (Spoel *et al*., 2003). Moreover, cytosolic NPR1 is required for JA- and ET-mediated soil-borne fungal resistance (Johansson *et al*., 2006). These studies suggest that NPR1 functions in diverse pathways of plant defence.

As an example of a monocotyledonous plant for which the entire genome sequence has been derived, rice (*Oryza sativa* L.) has been adopted as a model to study the molecular mechanisms of the defence response in cereal crops. However, by contrast with the extensive studies on disease resistance mechanisms in *Arabidopsis* and tobacco, very limited information is available on the molecular mechanisms of rice disease resistance, although a number of resistance genes have been cloned from the crop. Rice plants usually accumulate high levels of SA in leaves and shoots, and low SA levels in roots and suspension cells (Silverman *et al*., 1995; Chen *et al*., 1997). SA levels in 28 rice varieties have shown a correlation with generalized resistance to blast caused by *Magnaporthe grisea* (Silverman *et al*., 1995), and probenazole (PBZ, a type of SA analogue) treatment enhances resistance to rice blast (Sakamoto *et al*., 1999). Moreover, transgenic rice expressing the *nahG* gene, encoding a salicylate hydroxylase, fails to accumulate SA and shows an increased disease susceptibility to rice blast with runaway cell death-like lesions (Yang *et al*., 2004). These results suggest that SA may also play a role as a defence signal in rice. Over-expression of *NPR1* and the rice *NH1* gene (*NPR1 homologue 1*) also enhances disease resistance in transgenic rice (Chern *et al*., 2001, 2005b). Together with the functionally identified NPR1/NH1 interactor genes in rice (Chern *et al*., 2001, 2005a; Fitzgerald *et al*., 2005), these studies indicate that rice has probably evolved an SA-dependent pathway that shares downstream components with the SA-dependent pathway well established in *Arabidopsis*. However, more detailed research would shed light on the mechanism of rice *NPR1* homologue-mediated defence signalling, and provide a practical strategy for the improvement of disease resistance in the model cereal crop.

In this paper, we provide a systemic study of the rice *NPR1* homologues. We show that the rice homologous gene, *NH1*,

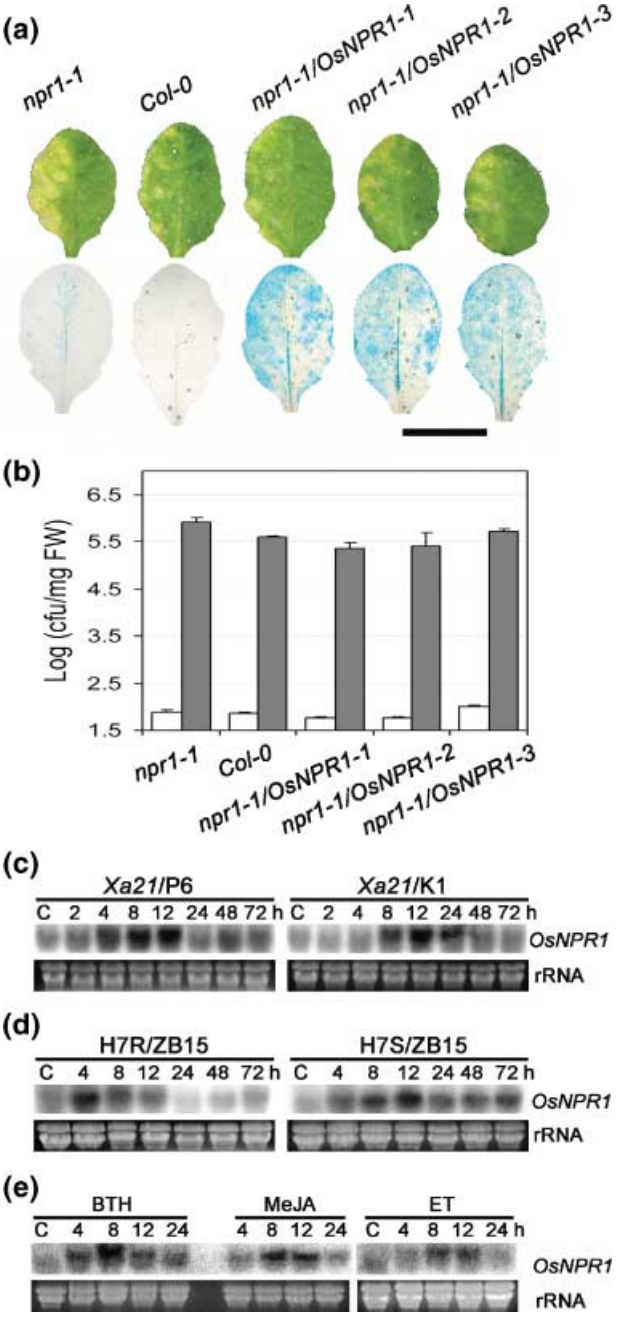

Figure 1 Complementation of the *Arabidopsis npr1-1* mutant by *OsNPR1*, and *OsNPR1* induction by pathogens and defence molecules. (a) Leaves of Col-0, *npr1-1* and three *npr1-1* plants transformed with *OsNPR1* (*npr1-1/OsNPR1*) and inoculated with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 for 4 days, bar: 1 cm; β-glucuronidase (GUS) staining was detected in these plant leaves. (b) Growth of *Pst* DC3000 in leaves of Col-0, *npr1-1* and three *npr1-1/OsNPR1* lines, indicating that the *OsNPR1* transgene restored *NPR1* function. (c–e) Induction of *OsNPR1* in the *Xa21–Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and rice–*Magnaporthe grisea* interactions, and by benzothiadiazole (BTH), methyl jasmonate (MeJA) and ethylene (ET). *Xa21* is resistant to P6 and susceptible to K1; H7R and H7S are resistant and susceptible to ZB15, respectively.

is the rice *NPR1* orthologue (hereafter called *OsNPR1*) through the complementation of the *Arabidopsis npr1-1* mutant. Over-expression of the wild-type and site-mutated OsNPR1 proteins results in increased disease resistance in transgenic rice. The OsNPR1 protein is located in the cytoplasm and moves into the nucleus by redox change; mutations in the conserved cysteine residues lead to the constitutive localization of OsNPR1 in the nucleus. Intriguingly, the *OsNPR1*-mediated disease resistance is associated with a penalty of enhanced herbivore susceptibility, and the nucleus-localized mutant proteins abolish this undesirable agronomic effect, providing a practical approach to engineer disease resistance without disturbing the insect resistance machinery probably mediated by JA in rice.

Results

Rice has five *NPR1*-like homologous genes

Through BLASTP search using the conserved ankyrin repeat domain of the *Arabidopsis* NPR1 protein, five *NPR1*-like genes were retrieved from the rice genome databases, named *OsNPR1* (same as *NH1*, accession no. DQ450948; Chern *et al.*, 2005b), *OsNPR2* (same as *NH2*, accession no. DQ450950; Chern *et al.*, 2005b), *OsNPR3* (accession no. DQ450952), *OsNPR4* (accession no. DQ450954) and *OsNPR5* (accession no. DQ450956). Phylogenetic analysis of NPR1-like proteins from diverse plants showed that OsNPR1 is the closest member of the rice family to NPR1 (Figure S1, available as Supplementary material). Through the prediction of protein localization, we found that OsNPR1 and OsNPR3, similar to NPR1, have nucleus location signals at their C termini (data not shown).

OsNPR1 complements the *Arabidopsis npr1-1* mutant and is induced by pathogens and defence molecules

We isolated the native promoter of the *Arabidopsis NPR1* gene, fused it to the *OsNPR1* full-length complementary DNA (cDNA) and transferred the fusion gene into the *Arabidopsis npr1-1* background (Cao *et al.*, 1994). Through this genetic approach, we showed that *OsNPR1* is a likely rice orthologue of *NPR1* because it restores the expression of the *BGL2-GUS* reporter gene and the basal resistance to the virulent pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 in the mutant when expressed in the *npr1-1* background (Figure 1a,b).

It has been shown that *NPR1* is involved in the resistance response mediated by some *R* genes (Cao *et al.*, 1994;

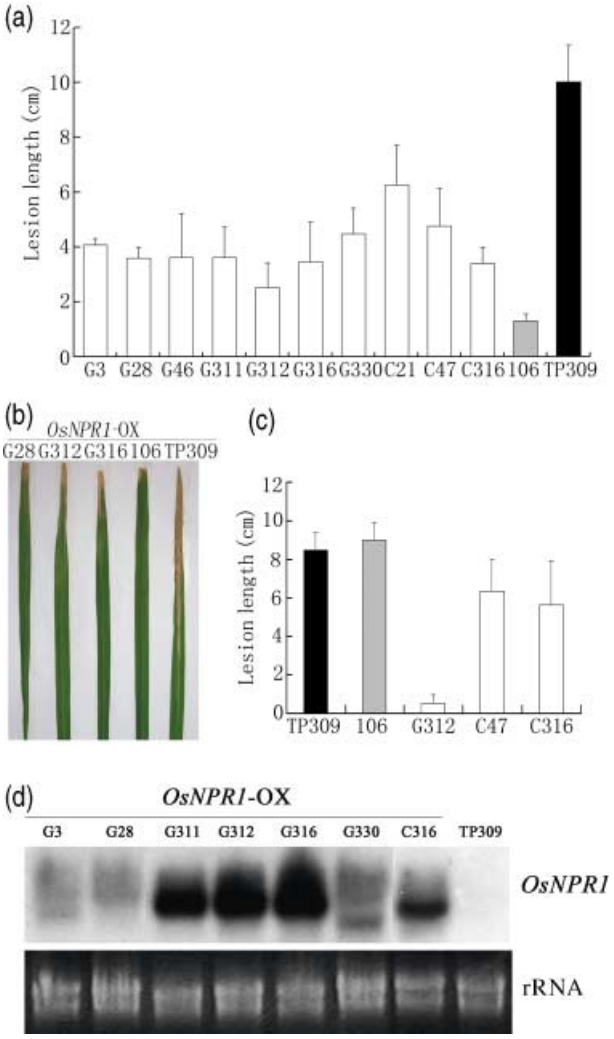

Figure 2 *OsNPR1* enhances resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). (a, b) Lesion lengths of 10 independent *OsNPR1*-OX T1 lines inoculated with *Xoo* P6, *Xa21*-transformed line 106 and wild-type TP309 as control. (c) Lesion lengths of three *OsNPR1*-OX T1 lines inoculated with *Xoo* K1, 106 and TP309 as control. Note that K1 is virulent to 106. (d) *OsNPR1* expression levels in the *OsNPR1*-OX lines. G, genomic DNA; C, complementary DNA.

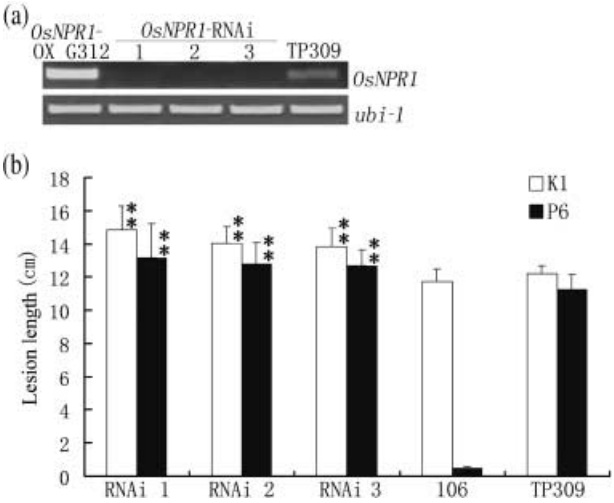

Figure 3 Disease resistance in *OsNPR1*-RNAi plants. (a) Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of the *OsNPR1* transcript in three *OsNPR1*-RNAi T1 lines, the wild-type and *OsNPR1*-OX G312 as control. (b) Lesion lengths of the three RNAi T1 lines inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) P6 and K1, with TP309 and 106 as controls, showing that RNAi enhances disease susceptibility. **Significant difference at $P < 0.01$ compared with the wild-type.

Delaney *et al*., 1995; Glazebrook *et al*., 1996; Shah *et al*., 1997). We examined the expression of *OsNPR1* in two *R*-mediated specific resistance responses in rice lines: the transgenic line 106 carrying the *R* gene *Xa21* that confers resistance to bacterial blight *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain P6 (Song *et al*., 1995), and the near-isogenic lines H7R and H7S with or without the *R* gene *Pir1* that confers resistance to rice blast (*M. grisea*) race ZB15 (He *et al*., 2000). In both pathosystems, *OsNPR1* was more rapidly induced in the incompatible (resistant) interactions than in the compatible (susceptible) interactions (Figure 1c,d). In addition, similar to *NPR1* that is induced by defence molecules such as SA and JA, transcript levels of *OsNPR1* were elevated by benzothiadiazole (BTH), an SA analogue, methyl jasmonate (MeJA) and ET (Figure 1e). These results suggest an essential role for *OsNPR1* in rice disease resistance.

OsNPR1 over-expression increases resistance and RNA interference (RNAi) enhances susceptibility to *Xoo*

We generated transgenic rice (*OsNPR1*-OX) over-expressing the *OsNPR1* genomic coding region and the full-length cDNA. Both genomic DNA (G) and cDNA (C) transgenic lines exhibited significantly enhanced resistance to *Xoo* strains P6 (Figure 2a,b) and K1 (Figure 2c); the latter is a virulent strain to *Xa21* (Song *et al*., 1995). Similar to previous reports (Cao *et al*., 1998; Chern *et al*., 2001, 2005b), *OsNPR1*-mediated disease resistance was displayed with a dose-related manner of transgene expression (Figure 2d). Furthermore, we generated *OsNPR1* 'knockout/knockdown' lines with RNAi, which did not accumulate the *OsNPR1* transcript. These lines were more susceptible to both *Xoo* strains compared with the wild-type (Figure 3a,b), indicating that *OsNPR1* is truly involved in basal resistance to *Xoo*. We did not observe similar enhanced resistance to *Xoo* in the transgenic lines over-expressing two other *NPR1*-like genes, Os*NPR2* and *OsNPR3* (Figure 4), although both genes were also induced in the *Xa21*- and *Pir1*-mediated resistance responses, and in

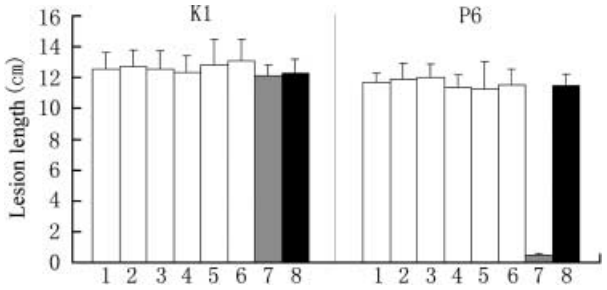

Figure 4 Inoculation of *OSNPR2*-OX and *OsNPR3*-OX plants. Lesion lengths of *OsNPR2*-OX (1–3) and *OsNPR3*-OX (4–6) T1 lines inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) K1 and P6, with 106 (7) and TP309 (8) as controls. Note that *OsNPR2* and *OsNPR3* do not confer disease resistance.

responses to BTH, MeJA and ET (data not shown). Therefore, we conclude that *OsNPR1* is a true *NPR1* orthologue that functions in rice disease resistance. Although the constitutive expression of *NPR1* enhances resistance to both bacterial and fungal pathogens in *Arabidopsis* and wheat (Cao *et al*., 1998; Friedrich *et al*., 2001; Makandar *et al*., 2006), we did not observe increased resistance to rice blast in these *OsNPR1* transgenic lines (data not shown), suggesting that the resistance mechanism to *Xoo* is distinct from that to rice blast, consistent with the different induction of 12 rice defence-related (*DR*) genes in the rice–*Xoo* and rice–*M. grisea* interactions reported previously (Wen *et al*., 2003). Further support to the hypothesis that rice adopts distinct defence mechanisms against bacterial and fungal pathogens is provided by microarray analysis of the *Xa21*- and *Pir1*-mediated resistance, using a gene chip containing 10 254 rice cDNAs representing 9240 unique genes. This showed only a limited overlap of defence responses to *M. grisea* and *Xoo* (Li *et al*., 2006; microarray data available at http://www.nlpmg.labs.gov.cn/Signal/Ricearray/Supplemental1.xls).

Subcellular localization of OsNPR1-green fluorescent protein (GFP) and OsNPR1(2CA)-GFP

To further understand the mechanism of *OsNPR1* function, we produced transgenic rice (*OsNPR1-GFP*-OX) over-expressing the *OsNPR1-GFP* fusion (Figure 5f), and found that the OsNPR1-GFP fusion protein was localized in the cytoplasm, similar to NPR1, although the rice cells usually showed obscure GFP fluorescence (Figure 5a). Treatment with the strong reducing reagent dithiothreitol (DTT; 100 mM) resulted in the movement of OsNPR1-GFP into the nucleus (Figure 5b), suggesting that the OsNPR1-GFP oligomer was converted to the monomer by a change in cellular redox

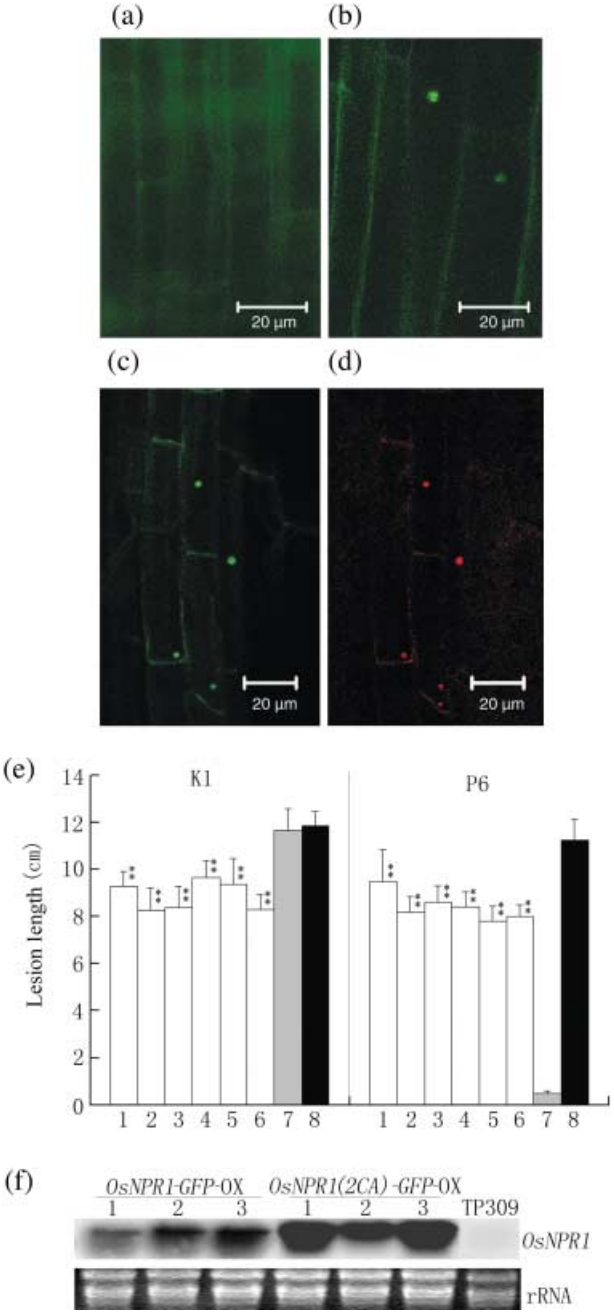

Figure 5 Subcellular localization of OsNPR1-green fluorescent protein (GFP) and OsNPR1(2CA)-GFP and disease resistance. (a) OsNPR1-GFP localized in the cytoplasm of rice root tip cells. (b) OsNPR1-GFP moved into the nucleus after treatment with dithiothreitol (DTT). (c) OsNPR1(2CA)-GFP accumulated in the nucleus of rice cells. (d) The same cells stained with propidium iodide (PI) to indicate the nucleus. (e) Lesion lengths of *OsNPR1-GFP*-OX (1–3) and *OsNPR1(2CA)-GFP*-OX (4–6) lines inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) K1 and P6, with 106 (7) and TP309 (8) as controls. **Significant difference at $P < 0.01$ compared with the wild-type. (f) Transcripts of *OsNPR1-GFP* and *OsNPR1(2CA)-GFP* were detected in these lines by Northern blot analysis.

potential. It is known that the two conserved cysteine residues (C82 and C216) are essential to the formation of the NPR1 oligomer through disulphide bonds, and that mutations in the residues cause constitutive monomerization and localization of NPR1 in the nucleus (Mou *et al*., 2003). The OsNPR1 protein also contains two conserved cysteine residues at positions 76 and 216 (C76 and C216). We mutated the two residues into alanine (C76A + 216A, 2CA) and generated transgenic rice lines *OsNPR1(2CA)-GFP*-OX over-expressing the mutant *OsNPR1(2CA)-GFP* fusion (Figure 5f). We found that OsNPR1(2CA)-GFP, similar to the mutant NPR1 (Mou *et al*., 2003), was localized in the nucleus (Figure 5c,d). The independent *OsNPR1-GFP*-OX and *OsNPR1(2CA)-GFP*-OX lines also exhibited resistance to *Xoo*, although weaker than *OsNPR1*-OX plants (Figures 2a,c and 5e). Most likely, the GFP fusion influences the effect of OsNPR1 on disease resistance.

Enhanced herbivore susceptibility associated with *OsNPR1*-mediated disease resistance

When grown in the glasshouse, *OsNPR1*-OX and *OsNPR1-GFP*-OX plants attracted more rice insect pests, such as acarids, than did wild-type and *OsNPR1(2CA)-GFP*-OX plants, leading to enhanced susceptibility to insect damage (data not shown). As *OsNPR1*-OX plants were slightly dwarf, as reported previously (Chern *et al*., 2005b), we compared the resistance of *OsNPR1-GFP*-OX and *OsNPR1(2CA)-GFP*-OX plants, which were morphologically normal and exhibited the same degree of resistance to *Xoo* (Figure 5e), with that of the wild-type, to rice white-backed planthopper (WBPH), *Sogatella furcifera* (Horvath), one of the most destructive rice insect pests in Asia. The number of WBPH on *OsNPR1-GFP*-OX plants was statistically greater than on wild-type plants at 30 days after infestation (DAI) when offspring nymphs were dominant, leading to higher WBPH growth rates (reproducibility) on these plants (Figure 6a,c). These results indicate that the increase in disease resistance resulting from cytoplasm-localized OsNPR1 is associated with a penalty of enhanced herbivore susceptibility.

OsNPR1 regulates defence-related genes but not SA and JA levels

The same function and subcellular localization pattern of OsNPR1 suggest that similar cellular activities, as mediated by NPR1 in *Arabidopsis*, may occur in rice cells. OsNPR1 may activate a similar SA-dependent pathway in rice that subsequently represses the JA signalling pathway, resulting in increased disease resistance and enhanced herbivore susceptibility in transgenic rice. We analysed the expression of *DR* genes in intact leaves of transgenic and wild-type plants. The SA-responsive gene *OsPR1b*, similar to the *Arabidopsis PR1*, was up-regulated by BTH and MeJA, and pretreatment with BTH inhibited MeJA induction of *OsPR1b* (Agrawal *et al*., 2000). We found that *OsPR1b* expression was greatly elevated in *OsNPR1(2CA)-GFP*-OX plants, but decreased in *OsNPR1*-RNAi plants, and not regulated in *OsNPR1*-OX and *OsNPR1-GFP*-OX plants (Figure 7a). This result demonstrates that the expression of *OsPR1b* is activated by the localization of OsNPR1 in the nucleus. Because NPR1 is involved in the antagonism of SA- and JA-mediated defence responses (Spoel *et al*., 2003; Durrant and Dong, 2004), we further examined the expression of JA-responsive genes. We found that several JA-responsive genes were down-regulated in the *OsNPR1*-OX and *OsNPR1-GFP*-OX transgenic rice lines. *JAI1*, a JA- and pathogen-induced gene identified in our microarray analysis of rice *DR* genes (Li *et al*., 2006), which encodes a putative jacalin protein, was quickly and strongly induced by MeJA (data not shown). In contrast with *OsPR1b*, JAI1 was repressed in *OsNPR1-GFP*-OX plants, but not in *OsNPR1(2CA)-GFP*-OX plants (Figure 7b). Similarly, *RCI-1*, a rice lipoxygenase gene induced by MeJA, BTH, INA and PBZ (Schaffrath *et al*., 2000), was repressed in *OsNPR1-GFP*-OX plants in which OsNPR1 accumulated in the cytoplasm (Figure 7c).

NPR1 can mediate the feedback regulation of SA accumulation in *Arabidopsis* (Delaney *et al*., 1995). To determine whether *OsNPR1* also elevates SA levels that lead to the repression of the JA pathway in rice, we measured SA and JA levels in these lines. However, rice usually contains high SA levels (Silverman *et al*., 1995), and we did not detect a consistent correlation between free and total SA levels, *OsNPR1* expression levels, protein subcellular localization and JA levels (Table 1). These results suggest that the stimulation of the SA pathway by *OsNPR1* inhibits JA signalling in rice, although significant changes were not detected in SA and JA levels. Therefore, our results suggest that rice has evolved an SA-dependent defence pathway similar to that in *Arabidopsis*, and that *OsNPR1* functions in the antagonistic control of SA- and JA-mediated responses occurring in the rice cytoplasm, probably through regulation of their signalling rather than their levels.

Localization of the mutant OsNPR1 in the nucleus abolishes herbivore susceptibility

The antagonistic cross-talk between the SA- and JA-mediated responses may explain the enhanced herbivore susceptibility

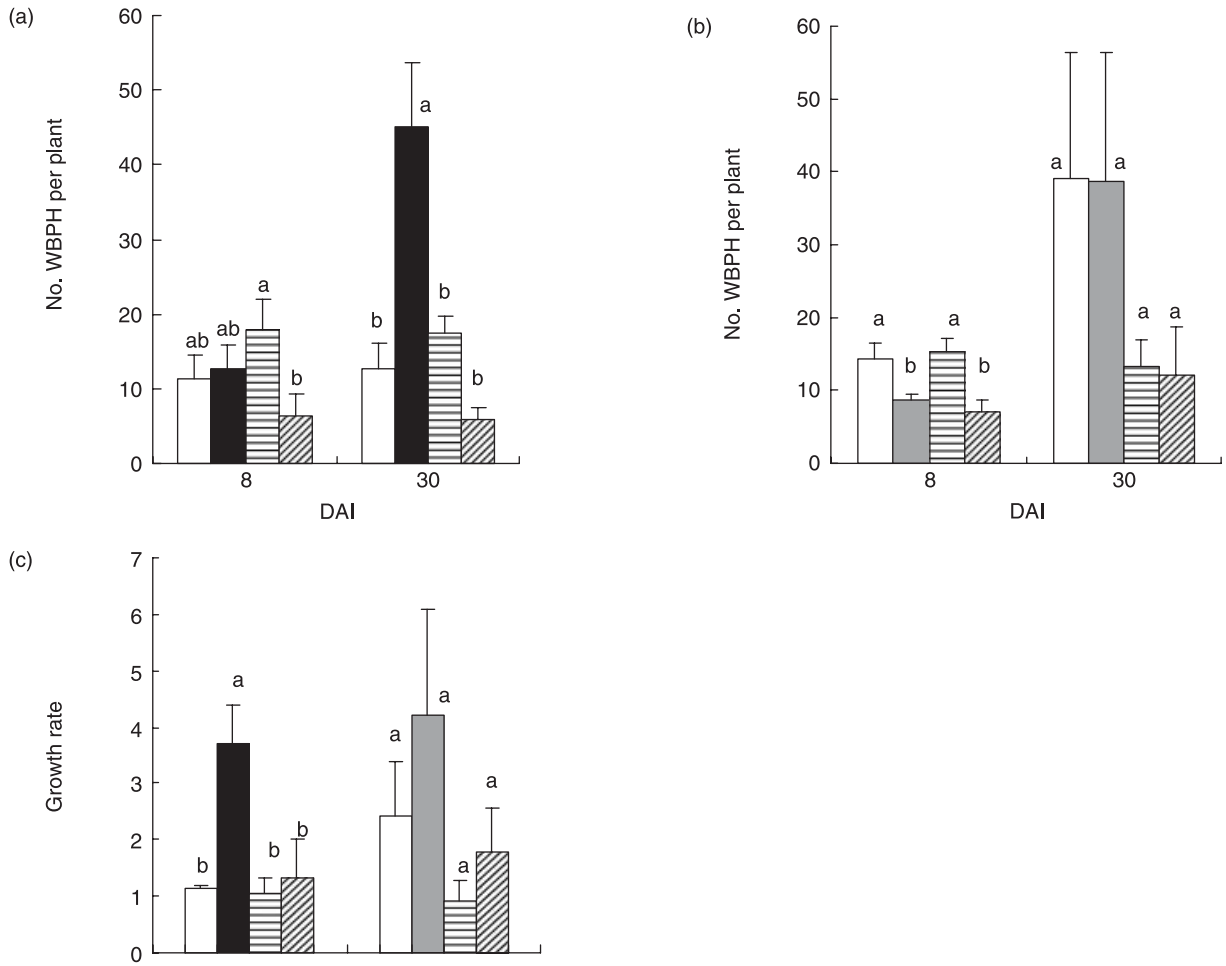

Figure 6 OsNPR1 subcellular localization affects insect resistance. (a) Resistance to the rice white-backed planthopper (WBPH) was compromised in the *OsNPR1-GFP*-OX plants. The number of WBPH on *OsNPR1-GFP*-OX (filled bar) was significantly greater than that on TP309 (open bar) at 30 days after inoculation (DAI) when offspring nymphs were dominant [$F_{3,11}$ = 12.565, P = 0.0021; analysis of variance (ANOVA) and least significant difference (LSD) comparison]. (b) Insect resistance was restored in the *OsNPR1(2CA)-GFP*-OX plants in independent assays. No difference was observed between the numbers of WBPH on *OsNPR1(2CA)-GFP*-OX (grey bar) and TP309 ($F_{3,11}$ = 2.059, P = 0.1842), except that the number of WBPH was significantly lower on *OsNPR1(2CA)-GFP*-OX than on TP309 at 8 DAI during the growth stage of adults ($F_{3,11}$ = 5.816, P = 0.0208). (c) The WBPH growth rate from 8 to 30 DAI was significantly higher on *OsNPR1-GFP*-OX than on TP309 ($F_{3,11}$ = 6.513, P = 0.0153), but there was no difference between *OsNPR1(2CA)-GFP*-OX and TP309 in independent assays ($F_{3,11}$ = 1.437, P = 0.3022). Data are means + standard error (n = 3). TN1 (horizontally shaded bar) and RHT1 (oblique shaded bar) were used as susceptible and resistant controls, respectively, to indicate successful insect feeding. Note that TN1 was seriously damaged by insects and dead at 30 DAI, leading to fewer nymphs.

in transgenic rice plants in which OsNPR1 accumulates in the cytoplasm (Figure 6a,c). Interestingly, our insect infestation experiment showed that, by contrast with *OsNPR1-GFP*-OX plants, *OsNPR1(2CA)-GFP*-OX plants accumulating the mutated OsNPR1 protein in the nucleus (Figure 5c,d) showed less infesting adults at 8 DAI, and the same number of offspring at 30 DAI, relative to wild-type plants (Figure 6b,c), indicating that the enhanced herbivore susceptibility was abolished in these plants. Consistent with this observation, *JAI1* and *RCI-1* were not down-regulated, or were less down-regulated, in these plants, indicating that the JA pathway was not affected, or was less affected, by mutant OsNPR1 localized in the nucleus. Therefore, the modification of the protein for nucleus localization abolishes the negative effect of *OsNPR1* on insect resistance.

Discussion

We have isolated rice *NPR1* homologous genes and have generated transgenic rice over-expressing these genes. Our results confirm that one of the rice homologues, *OsNPR1* (also known as *NH1*), is the rice orthologue that complements

Table 1 Salicylic acid (SA) and jasmonic acid (JA) levels in transgenic and wild-type plants

Lines	SA* (μg/g fresh mass)		JA† (μg/g fresh mass)
	Free SA	Total SA	
TP309 (control)	36.7450 ± 5.1950	40.1549 ± 2.7058	0.1378 ± 0.0054
OsNPR1-OX (G316)	–	–	0.2615 ± 0.0358
OsNPR1-OX (G312)	36.1606 ± 2.8442	40.4326 ± 0.8650	0.1344 ± 0.0082
OsNPR1-GFP-1	–	–	0.2338 ± 0.0217
OSNPR1-GFP-2	37.5440 ± 2.5441	39.0083 ± 1.5349	0.1519 ± 0.0155
OsNPR1-RNAi-1	33.7553 ± 1.7866	35.5659 ± 2.7258	0.1260 ± 0.0126
OsNPR1-RNAi-2	–	–	0.1177 ± 0.0178
OsNPR1(2CA)-GFP-1	38.8199 ± 6.6228	42.4871 ± 5.4490	0.0928 ± 0.0041
OsNPR1(2CA)-GFP-2	–	–	0.1354 ± 0.0095

GFP, green fluorescent protein; RNAi, RNA interference.
*Means ± standard error (n = 3)
†Means ± standard error (n = 5 or 6).

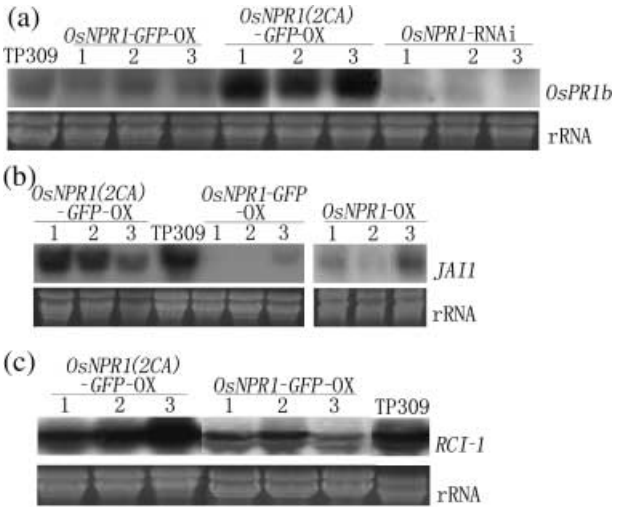

Figure 7 *OsNPR1* regulates defence-related gene expression. (a) *OsPR1b* was up- and down-regulated in *OsNPR1(2CA)-GFP*-OX and RNA interference lines, respectively. (b, c) The jasmonic acid (JA)-responsive genes, *JAI1* and *RCI-1*, were down-regulated in *OsNPR1-GFP*-OX and *OsNPR1*-OX plants compared with the wild-type.

the *Arabidopsis npr1* mutant. Over-expression of *OsNPR1* confers high levels of disease resistance to rice bacterial blight, whereas *OsNPR1* knockdown/knockout plants display more susceptibility to the bacterial pathogen. In contrast with a previous report (Chern *et al*., 2005b), we did not observe a reliable lesion-mimic phenotype on the leaves of all transgenic lines grown in the glasshouse or in paddy fields in Shanghai and Hainan Island at different latitudes (data not shown). Whether the difference in genetic background or growth conditions can explain these inconsistent results remains to be investigated.

We found that the wild-type OsNPR1 protein (in GFP fusion) was located in the cytoplasm, and moved into the nucleus on reduction caused by DTT treatment, whereas mutant OsNPR1(2CA) protein accumulated in the nucleus (Figure 5); this is similar to NPR1 in *Arabidopsis*. However, although rice generates much higher levels of endogenous SA than *Arabidopsis*, it does not constitutively activate the over-expressed OsNPR1 (OsNPR1-GFP) protein to move into the nucleus as in *Arabidopsis* (Mou *et al*., 2003). The same involvement in disease resistance and the same subcellular localization pattern of OsNPR1 in rice, as NPR1 in *Arabidopsis*, strongly suggest that similar cellular activities occur in rice. In rice, redox changes during defence activation or SAR-like responses lead to OsNPR1 monomerization and localization in the nucleus, which result in *PR* gene induction.

Little is known about the defence network or the true role of SA in rice disease resistance (Silverman *et al*., 1995; Yang *et al*., 2004). In particular, a model of SAR action has not been established for rice. The different expression of rice *DR* genes in these transgenic plants may provide a molecular clue as to how SA-mediated SAR is stimulated in rice. For example, *OsPR1b* and *JAI1* may be reliable markers for the activation of the SA- and JA-dependent defence pathways, respectively. In *OsNPR1*-OX and *OsNPR1-GFP*-OX plants, in which OsNPR1/OsNPR1-GFP is localized in the cytoplasm, the SA-responsive gene *OsPR1b* was not up-regulated (Figure 7a); however, OsNPR1(2CA)-GFP, with point mutations in OsNPR1, which is targeted to the nucleus, constitutively activated *OsPR1b*, similar to *Arabidopsis* (Mou *et al*., 2003). Interestingly, when OsNPR1 was localized in the cytoplasm, expression of the JA-responsive genes *JAI1* and *RCI-1* was inhibited; however, these genes were not affected, or only slightly affected, in

OsNPR1(2CA)-GFP-OX plants in which the mutant OsNPR1 was targeted to the nucleus (Figure 7b,c). It is known that NPR1 modulates the cross-talk between the SA- and JA-dependent pathways (Spoel *et al*., 2003). Our results further suggest that the antagonistic interaction between the SA and JA pathways could occur in the cytoplasm, probably through redox homeostasis that is regulated by both SA and JA signals, and that NPR1 and its orthologue proteins sense changes in redox status, as reported previously (Mou *et al*., 2003).

By contrast, transgenic rice plants over-expressing *OsNPR1* and GFP fusions, and *OsNPR1-RNAi*, do not exhibit significant changes in the levels of free and total SA, relative to *NPR1* in *Arabidopsis*. The fact that rice contains high levels of endogenous SA in different varieties may explain the divergence between the dicotyledonous and monocotyledonous model plants (Silverman *et al*., 1995). However, the possibility cannot be excluded that fine-tune regulation or components of the *NPR1*-mediated defence pathway may be somewhat different in rice compared with *Arabidopsis* (Chern *et al*., 2001), given the fact that *PR* genes are differently expressed in *Arabidopsis* and rice, and that over-expression of *OsNPR1* does not enhance resistance to blast fungus. Alternatively, the rice SA receptor (if any) might perceive the SA signal in a manner different from that in *Arabidopsis*. Thus, most probably, SAR-like disease resistance is activated against bacterial blight through SA signalling, which may be less dependent on the elevation of SA levels in rice cells. Consequently, JA levels are also not altered in an obvious manner; instead, expression of the JA-responsive genes is repressed in transgenic rice over-expressing *OsNPR1* and *OsNPR1-GFP*.

Rice bacterial blight is one of the most destructive diseases in rice, and disease resistance to this pathogen is of immense interest to rice breeders. However, as a result of the 'gene-for-gene' specificity and complexity of the natural pathogen population, breeding for rice blight resistance with *R* genes, such as *Xa21*, remains a challenge. Because of their conserved function in diverse plant species, *NPR1* genes, such as *NPR1* and *OsNPR1*, may be the first choice to produce transgenic crops for broad-spectrum disease resistance in agriculture. However, the enhanced herbivore susceptibility associated with transgenic rice expressing the wild-type *OsNPR1* gene greatly limits its agronomic value. As a solution to this problem, the subcellular targeting patterns of OsNPR1 greatly affect *DR* gene expression as well as herbivore resistance. We found that transgenic rice expressing the mutant OsNPR1 protein no longer displayed this penalty trait. Our study provides a practical approach to engineer rice broad-spectrum disease resistance to *Xoo* using the site-mutated *OsNPR1* gene, without disturbing the insect resistance machinery most probably mediated by JA in rice.

Experimental procedures

Plant materials and growth conditions

The *japonica* rice cultivar Taipei 309 (TP309) was used in rice transformation. The blast resistance near-isogenic lines H7R (resistant) and H7S (susceptible) were used in the rice–*M. grisea* interactions (He *et al*., 2000). The *Xa21* transgenic line 106 was provided by Dr Pamela Ronald (Dept. Plant Pathology, UC Davis, CA) (Song *et al*., 1995). All rice plants were grown in the glasshouse at 28–35 °C under sunlight. The *Arabidopsis* mutant *npr1-1* was supplied by Dr Xinnian Dong (Developmental, Cell and Molecular Biology Group, Dept. Biology, Duke University, Durham, NC) (Cao *et al*., 1994). *Arabidopsis* plants were grown in growth chambers at 21–25 °C, 14 h/10 h (day/night).

Phylogenetic analysis

The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for protein sequences homologous to NPR1. The NPR1-like sequences obtained were aligned using MEGA version 3.1 software, and the neighbour-joining tree was generated with the Poisson correction method. Bootstrap replication (500 replications) was used for statistical support for the nodes in the phylogenetic tree.

Gene cloning, plasmid construction and plant transformation

OsNPR1, *OsNPR2* and *OsNPR3* full-length cDNA and genomic DNA were isolated from TP309 and confirmed by sequencing; they were then inserted into the over-expression vector 35S-C1301 (provided by Dr Pamela Ronald). The *OsNPR1-GFP* fusion was made by in-frame fusion of the full-length *OsNPR1* cDNA with GFP (accession number U87973). The fusion gene was inserted into the vector 35S-C1301. Rice transformation was performed by an *Agrobacterium*-mediated method (Hiei *et al*., 1994). All rice transformants were identified by polymerase chain reaction (PCR) and GUS staining, and the second and third generations (T1 and T2) were used for all assays. The promoter region (2 kb upstream) of the *Arabidopsis NPR1* gene was fused with the full-length *OsNPR1* cDNA to generate the *AtNPR1::OsNPR1* fusion to complement the mutant *npr1-1*.

Site mutagenesis and RNAi of *OsNPR1*

Mutations of the conserved cysteines in OsNPR1 were generated using the Quickchange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), with the primers NC76AF (5'-CTGCTGGTGCACCGCGCCGTGCTCTCCGCGCGGAGC-3'), NC76AR (5'-GCTCCGCGCGGAGAGCACGGCGCGGTGCACCAGCAG-3'), NC216AF (5'-TGCAACAAATCTGCCATGAAACTGCTTGAAAGATGC-3') and NC216AR (5'-GCATCTTTCAAGCAGTTTCATGGCAGATTTGTTGCA-3'). The resultant mutant *OsNPR1(C76A + C216A)/2CA* was ligated in-frame to GFP to form the *OsNPR1(C76A + C216A)/2CA-GFP* fusion that

was inserted into 35S-C1301 for rice transformation. A 600-bp *OsNPR1* cDNA fragment with 3′ non-coding and partial coding regions was inserted into the RNAi (double-stranded RNA) vector 1300S (provided by Dr Yinong Yang, Dept. Plant Pathology, The Pennsylvania State University, PA) and transformed to TP309 to generate RNAi plants.

Subcellular localization of OsNPR1

The root tips of transgenic plants expressing the GFP fusions were observed directly with a confocal laser microscope (Zeiss LSM510; Carl Zeiss MicroImaging GmbH, Jena, Germany). For nucleus detection, root tips were stained for 30 min with 2 μg/mL propidium iodide (PI) in MES buffer [30 mM 2-(*N*-morpholino)-ethanesulphonic acid, 100 mM mannitol, pH 5.9], as described by Li *et al*. (2003).

Pathogen inoculation

Rice plants were inoculated with *Xoo* races P6 (incompatible to *Xa21*) and K1 (compatible to *Xa21*) using the leaf clip method (Song *et al*., 1995). After 14 days, the lesion lengths were recorded. Blast inoculation was performed as described previously (He *et al*., 2000). Infection of *Arabidopsis* with *Pst* DC3000 was performed as described by Shah *et al*. (1997). In brief, the virulent strain of *Pst* DC3000 was grown in King's B (KB) medium. Bacteria were collected and resuspended in 10 mM $MgCl_2$. For inoculation, 5-week-old leaves were infiltrated with a bacterial suspension [10^5 colony-forming units (cfu)/mL], and bacterial growth in inoculated leaves was measured at 0 and 4 DAI.

Treatment with defence molecules

BTH, MeJA and ET were sprayed on to H7R leaves at concentrations of 300, 100 and 100 μM, respectively. The treated leaves were harvested and immediately frozen in liquid nitrogen, followed by storage in a –80 °C freezer until RNA preparation.

RNA preparation and analysis

Total RNA was isolated from young leaves with TRIzol reagent, according to the manufacturer's protocol (Gibco-BRL, Gaithersburg, MD). Each 30 μg of total RNA was separated in 1.0% formaldehyde gel and transferred to Hybond-N^+ membranes (Amersham, Piscataway, NJ) for Northern analysis. The *OsNPR1*, *OsPR1b*, *JAI1* and *RCI-1* cDNA fragments were labelled with [α-^{32}P]dCTP using a random primer labelling kit (Takara, Shiga, Japan) for hybridization and autoradiography. Reverse transcriptase-PCR was performed to detect the *OsNPR1* transcripts in the RNAi plants.

SA and JA assay

Free and total SA were extracted from the third leaves of 3-week-old plants grown in a growth chamber at 25 °C/22 °C and 16 h/8 h (day/night), and analysed using an HP1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA), as described previously (Dewdney *et al*., 2000), with *o*-anisic acid (Sigma-Aldrich, St Louis, MO) as internal standard. Experiments were repeated three times. For JA assay, 40-day-old rice tissues were harvested and snap-frozen in liquid nitrogen, and stored at –80 °C. JA was extracted for analysis by gas chromatography-mass spectrometry (GC-MS) with the labelled internal standard D3-JA (kindly supplied by Ian T. Baldwin, Max Planck Institute for Chemical Ecology, Jena, Germany), as described previously (Lou and Baldwin, 2003), and JA levels were determined with five to six biological repeats.

Sogatella furcifera (Horvath) infestation and analysis

Five seedlings per line (15-day-old) were transplanted into pots (16×10 cm^2), together with the resistant and susceptible controls, Rathu Heenati (RHT) and Taichung Native 1 (TN1). Fifty days after transplanting (DAT), four pots with plants of the wild-type, RHT, TN1 and *OsNPR1-GFP*-OX or *OsNPR1(2CA)-GFP*-OX were arranged randomly in a tray with a Mylar cage. Five days later (55 DAT), 200 third to fourth instar WBPH [*Sogatella furcifera* (Horvath)] nymphs were released into the cage. The numbers of WBPH nymphs and adults were counted at 8 DAI, when the nymphs developed into adults, and 30 DAI, when offspring were produced and could not move from one plant hill to another, indicative of the reproduction of adults on a specific plant. The experiment was carried out as a randomized complete block design with three replicates in a screenhouse with a daily temperature of 25–30 °C. Analysis of variance (ANOVA) and least significant difference (LSD) comparisons were used to compare the mean rank of WBPH numbers on each rice line for each observation.

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Phylogenetic analysis of predicted NPR1-like proteins in plants. Amino acid sequences of NPR1-like proteins were obtained from the database (http://www.ncbi.nlm.nih.gov/), including those from *Arabidopsis*, rice, *Brassica napus*, *Lycopersicon esculentum*, *Helianthus annuus*, *Nicotiana tabacum* and *Beta vulgaris*. Accession numbers of the proteins are indicated. The scale bar indicates the number of amino acid substitutions per site. Bootstrap values in percentages are shown at each branch point.

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