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Involvement of OsNPR1/NH1 in rice basal resistance to blast fungus *Magnaporthe oryzae*

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Abstract Rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a major threat to worldwide rice production. Plant basal resistance is activated by virulent pathogens in susceptible host plants. *OsNPR1/NH1*, a rice homolog of *NPR1* that is the key regulator of systemic acquired resistance in *Arabidopsis thaliana*, was shown to be involved in the resistance of rice to bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* and benzothiadiazole (BTH)-induced blast resistance. However, the role of *OsNPR1/NH1* in rice basal resistance to blast fungus *M. oryzae* remains uncertain. In this study, the *OsNPR1* gene was isolated and identified from rice cultivar Gui99. Transgenic Gui99 rice plants harbouring *OsNPR1*-RNAi were generated, and the *OsNPR1*-RNAi plants were significantly more susceptible to *M. oryzae* infection. Northern hybridization analysis showed that the expression of pathogenesis-related (PR) genes, such as *PR-1a*, *PBZ1*, *CHI*, *GLU*, and *PAL*, was significantly suppressed in the *OsNPR1*-RNAi plants. Consistently, overexpression of *OsNPR1* in rice cultivars Gui99 and TP309 conferred significantly enhanced resistance to *M. oryzae* and increased expression of the above-mentioned PR genes. These results revealed that *OsNPR1* is involved in rice basal resistance to the blast pathogen *M. oryzae*, thus providing new insights into the role of *OsNPR1* in rice disease resistance.

Keywords Rice · *OsNPR1/NH1* · Basal resistance · Rice blast · *Magnaporthe oryzae* · PR genes

Abbreviations

TMV	Tobacco mosaic virus
SAR	Systemic acquired resistance
HR	Hypersensitive response
PR	Pathogenesis-related

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BTH	Benzothiadiazole
NPR1	Nonexpressor of PR genes 1
SA	Salicylic acid
INA	2,6-Dichloroisonicotinic acid
Xoo	*Xanthomonas oryzae pv. oryzae*
TF	Transcription factors
RT-PCR	Reverse-transcriptase polymerase chain reaction
RACE	Rapid Amplification of cDNA Ends
ORF	Open reading frame
dsRNA	Double-stranded RNA

Introduction

Rice (*Oryza sativa* L.) is one of the world's most important crops. Rice diseases pose a major year-on-year threat to rice production. The three major rice diseases, rice blast, bacterial blight, and sheath blight, cause significant yield loss and quality reduction, leading to significant economic and humanitarian problems. Among these three diseases, rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a most serious and recurrent problem in all rice-growing regions, and which destroys enough rice to feed 60 million people annually (Skamnioti and Gurr 2009).

Resistant cultivars, fungicides, and biotechnological methods have been developed to control rice blast disease. However, it is difficult to obtain resistant rice cultivars because of high pathogenic diversity. Fungicides are becoming less acceptable as they increase the potential for fungicide resistance in *M. oryzae*, and also conflict with the public concern for effects of fungicide residues on human health and environment (Marĭa et al. 2006).

Biotechnological methods are effective for providing resistance to rice blast. Quite a number of attempts were made by generating rice transformed with plant antifungal genes (Marĭa et al. 2006). However, the protection provided by a single antifungal gene is limited since the degree of resistance is relatively low (Marĭa et al. 2006). Most of the resistance dissipates after a few years of intensive agricultural use. In addition, rice is usually attacked by more than one kind of pathogen. Therefore, introducing genes that can elicit broad spectrum disease resistance into rice plants and developing novel strategies for achieving disease management are desirable.

Plants have two major types of disease resistance, basal resistance and resistance (*R*) gene-mediated resistance. Plant basal resistance is activated by virulent pathogens in susceptible host plants. The *R* gene-mediated resistance is activated upon indirect or direct recognition of an avirulence factor (Avr) or a pathogen effector by one of the protein products encoded by *R* genes, resulting in disease resistance and, usually, a hypersensitive response (HR) at the site of infection (Jones and Dangl 2006). The HR often triggers systemic acquired resistance (SAR), which coordinately induces expression of a set of pathogenesis-related (*PR*) genes, leading to a long-lasting enhanced resistance against a broad spectrum of pathogens (Durrant and Dong 2004).

SAR in dicots such as *Arabidopsis thaliana* and tobacco has been well characterized, and a set of PR genes has been identified as SAR marker genes (Durrant and Dong 2004; Grant and Lamb 2006). SAR-like mechanisms have also been reported in monocots. In rice, it was shown that benzothiadiazole (BTH) application enhances resistance to *Rhizoctonia solani* (Rohilla et al. 2002) and *M. oryzae* (Schweizer et al. 1999; Sugano et al. 2010). *Pseudomonas syringae* pv. *syringae* could induce systemic resistance of rice to *M. oryzae*(Smith and Metraux 1991).

NPR1 (nonexpressor of PR genes 1), also known as *NIM1* and *SAI1*, is a key regulator of the SA-mediated SAR pathway in *A. thaliana*. It has been documented that NPR1 regulates expression of *A. thaliana PR* genes by interacting with the TGA family members of basic leucine-zipper transcription factors. In non-induced cells, NPR1 exists as an oligomeric form in the cytoplasm. During SAR induced by SA or pathogen, it is converted into a monomer and moves into the nucleus where it interacts with TGA factors for activation of *PR* gene expression (Durrant and Dong 2004). The conformational changes of NPR1 are mediated by S-nitrosylation and thioredoxins (Tada et al. 2008). Recently, it was found that proteasome-mediated turnover of nuclear NPR1 protein plays an important role in modulating transcription of its target genes (Spoel et al. 2009).

It is also known that overexpression of *NPR1* in *A. thaliana*, tomato, apple, *Brassica napus*, carrot, cotton and citrus led to enhanced disease resistance to bacterial and/or fungal pathogens (Cao et al. 1998;

Friedrich et al. 2001; Lin et al. 2004; Malnoy et al. 2007; Potlakayala et al. 2007; Wally et al. 2009; Parkhi et al. 2010; Zhang et al. 2010). Overexpression of *NPR1* in rice resulted in enhanced resistance to the rice bacterial blight pathogen *Xanthomonas oryzae pv. oryzae* (*Xoo*) (Chern et al. 2001; Fitzgerald et al. 2004). Quilis et al. (2008) showed that the constitutive expression of the *NPR1* gene in rice conferred increased resistance against rice blast fungus *M. oryzae*.

NPR1 homologs have been identified in many plants, including economically important plants such as cabbage, broccoli, potato, corn, tobacco, tomato, canola, grapevine, wheat, soybean, apple, cotton, banana, cacao, and rice (Cao et al. 1998; Chern et al. 2005; Makandar et al. 2006; Malnoy et al. 2007; Potlakayala et al. 2007; Yuan et al. 2007; Endah et al. 2008; Zhang et al. 2008; Le Henanff et al. 2009; Sandhu et al. 2009; Shi et al. 2010). In rice, the *NPR1*-like gene *OsNPR1*/*NH1* had been isolated and functionally identified (Chern et al. 2005; Yuan et al. 2007).

It has been reported that overexpression of *OsNPR1* in rice resulted in enhanced resistance to the rice bacterial blight pathogen *Xoo* (Chern et al. 2005; Yuan et al. 2007). Shimono et al. (2007) reported that silencing of *OsNPR1* in rice impaired BTH-induced resistance to *M. oryzae*, but unfortunately no data were shown in this report. Sugano et al. (2010) recently showed that OsNPR1 plays essential role in BTH-induced blast resistance by using *OsNPR1* knockdown and overexpressing rice lines. However, the role of *OsNPR1* in rice basal resistance to *M. oryzae* is uncertain. Yuan et al. (2007) did not observe increased resistance to rice blast fungus in their *OsNPR1* overexpressing rice lines, and the interaction of the *OsNPR1* or *NPR1* overexpressing rice with rice blast fungus was not reported (Chern et al. 2001; 2005). Sugano et al. (2010) did not observe any significant difference in disease symptoms between wild-type rice and *OsNPR1* knockdown rice plants in the absence of BTH pretreatment after inoculation with *M. oryzae*.

In this work, we evaluated disease resistance of transgenic rice plants altered in expression of *OsNPR1*/*NH1* and their wild type plants to *M. oryzae* and carried out Northern analysis to detect the expression of PR genes in these plants after infection with *M. oryzae.* The results showed that *OsNPR1* plays a pivotal role in the basal resistance of rice to the fungal pathogen *M. oryzae*.

Materials and methods

Plant materials and pathogen strains

The experiments were performed with Indica rice cultivar Gui99 and with Japonica rice cultivar TP309. Gui99 is a very important restorer line of rice in Southern China. The hybrid rice derived from Gui99 was widely planted in the provinces of Guangxi, Guangdong, Hunan, Hainan, and Fujian in China, and also in Vietnam.

M. oryzae strain CHL0742 was employed to inoculate rice plants for evaluating resistance to blast disease. The rice cultivars Gui99 and TP309 are susceptible to *M. oryzae* CHL0742. *Xanthomonas oryzae* pv. *oryzae* strain 13751 was used in inoculation for assessing resistance to bacterial blight (Tang et al. 1996). Gui99 is moderately resistant to *Xoo* strain 13751. TP309 is susceptible to *Xoo* strain 13751.

RNA and DNA isolation

Total RNA was isolated from young leaves of rice with Trizol reagent (Invitrogen, San Diego, CA, USA) according to the method provided by the supplier. Genomic DNA was isolated from rice by using the cetyltrimethyl ammonium bromide (CTAB) method.

Cloning of the partial sequence of *OsNPR1* cDNA from rice cultivar Gui99

To obtain a partial sequence of the rice *OsNPR1* cDNA, the seedlings of 4-week-old rice cultivar Gui99 were sprayed with 0.3 mM BTH to induce the expression of the *OsNPR1* gene, and the total RNA was isolated from the treated rice 2 days after treatment. One microgram of total RNA was reverse transcribed at 42°C for 1 h in a 20 μl final volume reaction with Primescript RTase (Takara, Biotech Co., Ltd, Dalian, China). A primer pair, RiF2 and RiR2 (Table S1), were designed according to *NPR1*-like gene of Japonica group genomic DNA (GenBank accession No. AP002537). The PCR was performed with primers RiF2 and RiR2 under the following

conditions: one cycle of 2 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 45°C, 30 s at 72°C. A DNA band of approximately 230 bp (the expected size) was recovered and sequenced.

Southern analysis

For *OsNPR1* gene isolation from rice cultivar Gui99, genomic DNA (10 μg) from rice Gui99 was digested with *Bam*HI, *Eco*RI or *Eco*RI and *Bam*HI. The digested DNA was separated on a 1% agarose gel. After blotting onto the Hybond N^+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), filters were hybridized with the 228 bp *OsNPR1* cDNA labeled with α-^{32}P-dCTP by the Prime-A-Gene labeling system (Promega, Madison, WI, USA). Hybridization was carried out following standard procedures (Sambrook and Russell 2001). The hybridized filter was washed in high stringency conditions.

For transgenic plant identification, 10 μg of genomic DNA extracted from transgenic plants and wild-type plants were digested with *Hind*III. The 422 bp *Bam*HI/*Spe*I DNA fragment released from digestion of plasmid pCAMBIA1301-*OsNPR1*i (for *OsNPR1*-RNAi plants) and 3.1 kb *Eco*RV/*Xho*I DNA fragment recovered from the digestion of plasmid pCAMBIA1301-UbiN-*OsNPR1* (for *OsNPR1* over-expressing plants) were labeled with α-^{32}P-dCTP as probes. Hybridization was carried out as described above.

Construction of a partial library of rice cultivar Gui99, isolation and identification of rice Gui99 *OsNPR1* gene

Rice Gui99 genomic DNA was digested with *Eco*RI and *Bam*HI. The digested DNA was separated on 1% agarose gel, and the approximately 7.4 kb DNA fragments were recovered from the gel. The recovered DNA fragments were ligated into pGEM3zf(+) and a library containing *ca.* 12,000 clones was constructed. Colonies of the library were replica plated onto a nylon membrane and incubated on Luria-Bertani (LB) agar plates at 37°C for 12 h. The genomic library was screened for the *OsNPR1* gene using in situ hybridization following standard procedures (Sambrook and Russell 2001) and the 228 bp *OsNPR1* cDNA mentioned above was used as probe. Positive clones were routinely sequenced in both directions by an automated ABI 373 DNA Sequencer (Applied Biosystems).

Cloning and identification of Gui99 full length *OsNPR1* cDNA gene

For 3′ RACE, two specific primers, RACE3-1 and RACE3-2, were designed (Table S1). First-strand cDNA was synthesized from 5 μg of total RNA with the oligo d(T) adaptor primer (AP) according to the protocol of the 3′ RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, USA). 3′ RACE amplification was performed on 2 μl of the cDNA synthesis reaction with primers AUAP (GIBCO) and RACE3-1, semi-nested PCR was then carried out with AUAP and RACE3-2. The single PCR product was recovered and sequenced.

Based on the sequence of the 3′ RACE product, specific primers RACE5-1 and RACE5-2 were designed (Table S1) and synthesized to amplify the 5′ end of cDNA. For 5′ RACE, the cDNA was synthesized using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). 5′ RACE amplification was performed on 2.5 μl of 5′-ready-cDNA with Universal Primer Mix(UPM, Clontech) and RACE5-1, then the nested PCR was carried out with Nested Universal Primer A (NUP, Clontech) and RACE5-2. The resulting single PCR product containing the full-length *OsNPR1* cDNA was cloned into pGEM-T Easy vector and transformed into *E. coli* (DH5α). The inserts in three independent colonies were sequenced and compared with genomic sequences. The insert, which was identical with the Gui99 genomic *OsNPR1* gene, was selected for further study.

Plasmid construction and plant transformation

To construct the *OsNPR1*-RNAi rice expression vector pCAMBIA1301-*OsNPR1*i, the first intron of the potato GA20 oxidase gene (199 bp) was amplified with primer intF1 and intF2 (Table S1). The PCR product was digested with restriction enzymes *Bam*HI/*Sma*I and the recovered DNA fragment was inserted into the corresponding sites in the pCAMBIA1301-UbiN vector, between the Ubiquitin promoter and the NOS terminator (Helliwell and Warterhouse 2003). Part of the *OsNPR1* gene from

nucleotide 667 to 1,088 of the cDNA ORF (422 bp) was amplified by PCR using two pairs of primers, RiSF1 and RiSR1, RiAnF1 and RiAnR1 (Table S1) respectively, which have the same sequence but with different restriction sites at both ends. The amplified fragment with *Bam*HI/*Spe*I sites after digestion was inserted into a site upstream of the intron, while the fragment having *Sac*I/*Sma*I sites was inserted into a site downstream of the intron.

To construct a plasmid for overexpression of *OsNPR1*, a recombinant plasmid named pGXN4834 was obtained by cloning a 4834 bp *Xba*I/*Xho*I DNA fragment, which contains the full-length *OsNPR1* gene, into vector pBluescript KS(+). A primer pair, NPR1S and NPR1A (Table S1), was used to amplify the 188 bp DNA sequence from the start codon to the first restriction enzyme *Sma*I site on the first exon of *OsNPR1*. A restriction enzyme *Xba*I site was added at the left-hand end of the PCR product. The PCR product was digested with *Xba*I/*Sma*I and the recovered DNA fragment was used to replace the 597 bp *Xba*I/*Sma*I fragment in pGXN4834 to yield plasmid pGXN4432. A 4432 bp *Xba*I/*Xho*I DNA fragment was recovered after digestion of the plasmid pGXN4432 and was further treated with T4 DNA polymerase to generate blunt ends. The DNA fragment with blunt ends was cloned into the *Sma*I site of vector pCAMBIA1301-UbiN in the right orientation to produce the expression vector pCAMBIA1301-UbiN-*OsNPR1*. The full-length genomic DNA of *OsNPR1,* from the start codon to 192 bp downstream of the stop codon*,* was placed under the control of the maize ubiquitin promoter.

The resulting pCAMBIA1301-*OsNPR1*i and pCAMBIA1301-UbiN-*OsNPR1* were transferred into *A. tumefaciens* strain EHA105 by triparental conjugation. The media for rice tissue culture and transformation are listed in Table S2. Two-week-old seed calli of cultivar Gui99 were transformed with pCAMBIA1301-*OsNPR1*i, and Gui99 and TP309 were transformed with pCAMBIA1301-UbiN-*OsNPR1* independently, by soaking the calli with *A. tumefaciens* suspensions in AAM-AS medium and co-cultivating for 2–3 days in CC-AS medium. The calli were then placed on GS1 medium for selection for 2–3 weeks. Resistant calli were transferred to GS2 medium for a further selection for another 1–2 weeks, and transferred to GF medium for differentiation. Plantlets were then transferred to ½ MS medium to induce root formation.

The hygromycin-resistant plants were preliminarily identified by PCR analyses. Genomic DNA isolated from hygromycin-resistant and wild-type plants were used as PCR templates. The primers are shown in Table S1. For identifying *OsNPR1*-RNAi hygromycin-resistant plants derived from Gui99, two pairs of primers were used. The primer pair UBRNF and IntR was used to amplify the fragment containing the partial sequence of the ubiquitin promoter, the sense insertion of *OsNPR1*, and the complete intron of potato GA20 oxidase gene. The other primer pair, IntF and RNNOSR, was employed to amplify the DNA containing the whole intron, the anti-sense insertion of *OsNPR1*, and partial sequence of the NOS terminator. For characterizing *OsNPR1*-over-expressing hygromycin-resistant plants generated by transformation of Gui99 and TP309, a primer pair, RNPR1F and NOSR, was used to amplify a DNA fragment containing a partial sequence of *OsNPR1* and a partial sequence of the NOS terminator. Another primer pair, hptF and hptR, was employed to amplify the hygromycin-resistant gene.

RT-PCR analysis

For RT-PCR analysis of the expression of rice genes, total RNA was extracted from 4-week-old rice leaves and treated with DNase I (Takara). First-strand cDNA was synthesized with Primescript RTase (Takara). PCR on serial dilution of cDNA was performed at 56°C and 30 cycles to define semi-quantitative conditions that resulted in amplification linear to RNA amounts. The experiments were performed three times with similar results. Primers used for amplification reactions were NH1F/NH1R for *OsNPR1*, NH2F/NH2R for *OsNPR2*, NH3F/NH3R for *OsNPR3*, NH4F/NH4R for *OsNPR4*, and NH5F/NH5R for *OsNPR5* (Table S1). Analysis of the rice *Actin* gene was included as a control using the primer pair ActF/ActR.

Pathogen inoculation and disease evaluation

For blast fungus infection, *M. oryzae* strain CHL0742 was employed to inoculate Gui99 and TP309 and their derived lines. Rice plants were maintained in a greenhouse on a 28°C day/26°C night temperature

cycle with a 14 h daytime period. For 10 mM BTH treatment, the commercial product Bion (Ciba-Geigy GmbH, Germany) was used for foliar spray on the three-week-old rice plants. The mock treatment was foliar spray with distilled water. For inoculations, three-week-old rice seedlings were sprayed with fungal conidial spores at a concentration of 2.5×10^5 spores ml^{-1} containing 0.02% Tween-20. After incubation in a dew chamber at 22°C for 24 h, rice seedlings were moved to a greenhouse with 14 h of light. Disease was recorded as one of the ten levels 7 days post inoculation using a scoring system as described by the Standard Evaluation System for Rice (IRRI 2002; http://www.knowledgebank.irri.org/ses/SES.htm) as follows: level 0, no lesions observed; level 1, small brown specks of pin-point size or larger brown specks without sporulating center; level 2, small roundish to slightly elongated, necrotic gray spots, about 1–2 mm in diameter, with a distinct brown margin; level 3, lesion type is the same as in scale 2, but a significant number of lesions are on the upper leaves; level 4, typical susceptible blast lesions 3 mm or longer, infecting less than 4% of the leaf area; level 5, typical blast lesions infecting 4–10% of the leaf area; level 6, typical blast lesions infection 11–25% of the leaf area; level 7, typical blast lesions infection 26–50% of the leaf area; level 8, typical blast lesions infection 51–75% of the leaf area and many leaves are dead; level 9, more than 75% leaf area affected. Ten leaves were scored for each line in each treatment and each treatment was carried out in triplicate per experiment. Disease index for each treatment was calculated by using the formulae: $\text{Disease index}(\%) = [\sum(\text{Number of disease leaves} \times \text{corresponding disease level})/(\text{total number of tested leaves} \times \text{the highest disease level})] \times 100$.
Average disease index was calculated and statistically analyzed by a *t* test.

Four-week-old rice seedlings were inoculated with *Xoo* strain 13751 (Tang et al. 1996). The bacterial suspensions at 0.001 optical density units at 600 nm (approximately 1×10^6 cfu ml^{-1}) were inoculated into rice leaves by the leaf clipping method. Disease evaluation was performed by measuring the lesion length of 20 randomly chosen leaves of each line, 14 days post inoculation.

Northern hybridization

Leaf samples of rice were harvested at 0, 6, 12, 18, 24, and 48 h after pathogen inoculation, deep frozen with liquid nitrogen, and kept at −80°C until use. Total RNA (15 μg) isolated from each sample was separated on a 1.2% agarose gel containing formaldehyde followed by transfer onto a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech). Probes were labeled with radioactive α-^{32}P-dCTP according to the protocol of the Prime-a-Gene Labeling System (Promega). Northern hybridization was carried out following standard procedures (Sambrook and Russell 2001). Primers used for amplifying DNA probes for the following genes are listed in Table S3: rice actin (X15865), *PR-1a* (AJ278436), *PAL* (phenylalanine ammonia lyase, X87946), *POD* (peroxidase, AF014467), *PBZ1* (probenazole-inducible 1, AF395880), *CHI* (chitinase, AF296279), *GLU* (1,3-β-glucanase, AB027428), and *OsNPR1* (DQ450948).

Results

Isolation and identification of the *OsNPR1* gene from rice cultivar Gui99

In rice, there are five NPR1-like proteins encoded by six genes in the genome. Protein OsNPR5/NH5 is encoded by two duplicate genes which are recently duplicated genes because of chromosomal segmental duplication (Yuan et al. 2007; Bai et al. 2011). To isolate the *OsNPR1* gene from rice cultivar Gui99, we first obtained a 228 bp cDNA fragment by reverse-transcriptase polymerase chain reaction (RT-PCR) using primer pair, RiF2 and RiR2 (Table S1). The cDNA fragment was sequenced and found to be identical with the *OsNPR1* cDNA from the Indica cultivar-group (GenBank accession No. AY923983). Using the fragment as probe, the *OsNPR1* gene was located on a 7.4 kb *Eco*RI/*Bam*HI fragment of the rice cultivar Gui99 genome by Southern analysis (Figure S1). A DNA library containing *ca* 12,000 clones whose insert size were about 7.4 kb was constructed from complete digestion of rice Gui99 total DNA with *Eco*RI and *Bam*HI. The 228 bp *OsNPR1* cDNA

fragment was used to screen the library and a positive clone was identified (Figure S1) and its insert was sequenced. The insert was 7,417 bp in length and contained the full-length *OsNPR1* gene (GenBank accession No. GU722159), which shared 98% identity with the rice Japonica group *OsNPR1* gene (GenBank accession No. AP002537).

The full-length *OsNPR1* cDNA from rice Gui99 was identified by the Rapid Amplification of cDNA Ends (RACE) method. The *OsNPR1* cDNA was 2,040 bp long and contained a 1,749 bp open reading frame (ORF) (GenBank accession No. GU722160) encoding a protein of 582 amino acids. There was a 5′ untranslated region of 15 bp upstream from the start codon, and the coding region was followed by 3′ untranslated region of 276 bp downstream from the stop codon TGA. The *OsNPR1* cDNA gene was identical with the sequence of Gui99 genomic *OsNPR1* gene and shared 100% and 99% homology with the *OsNPR1* cDNA in the Indica cultivar-group (GenBank accession No. AY923983) and the Japonica cultivar-group (GenBank accession No. DQ450947), respectively.

Generation of *OsNPR1*-RNAi and *OsNPR1*-overexpressing plants

RNAi, a double-stranded RNA (dsRNA)-induced gene-silencing phenomenon, has been widely used as an efficient tool to analyze gene function in a range of organisms. In plants, RNAi is often achieved through transgenes that produce hairpin RNA (Kusaba 2004). In this study, to construct *OsNPR1*-RNAi plasmid, a 422 bp fragment (nucleotide 667 to 1,088) of the *OsNPR1* cDNA ORF, was selected to be amplified based on the rule of avoiding blocks of sequence identity of more than 20 bases between the RNAi construct and non-target gene sequences (Helliwell and Warterhouse 2003). Sequence alignment demonstrated that this fragment shares the highest homology with *OsNPR3* at 57.1% and has only one longest ungapped homology region of 17 bp with *OsNPR5* (Figure S2). The 422 bp *OsNPR1* sequence was placed on either side of the first intron of potato GA20 oxidase gene in an inverted repeat, thus forming an RNA with a hairpin-loop structure in the pCAMBIA1301-UbiN vector (Fig. 1a).

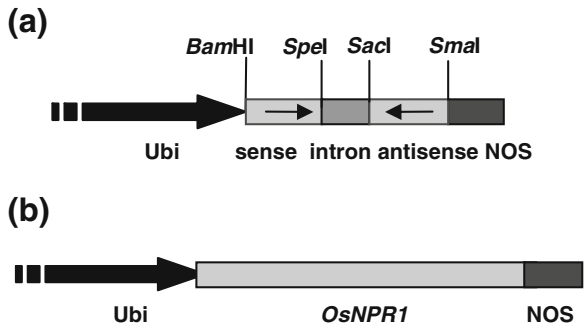

Fig. 1 Schematic representation of rice expression vectors. **a** Structure of *OsNPR1*-RNAi rice expression vector pCAMBIA1301-*OsNPR1*i. Ubi, maize ubiquitin promoter; sense, sense insert of *OsNPR1* cDNA ORF partial sequence from nucleotide 667 to 1,088 bp; Intron, the first intron of potato GA20 oxidase gene; antisense, antisense of the above *OsNPR1* sense sequence; NOS, NOS terminator. **b** Structure of rice *OsNPR1* overexpression vector pCAMBIA1301-UbiN-*OsNPR1*. Ubi, maize ubiquitin promoter; NOS, NOS terminator

The *OsNPR1*-RNAi construct was transformed into callus derived from rice cultivar Gui99 via *Agrobacterium*-mediated transformation. Six T1 hygromycin-resistant transgenic lines were analyzed by Southern hybridization. Apart from the common band of 3.9 kb in both transgenic and untransformed control plants, five transgenic lines gave one additional hybridizing band and the other one gave two additional hybridizing bands, which confirmed the integration of the transgene in all six lines, and indicated that *ca* 83% of the transgenic lines carried a single copy of the integrated transgene (Figure S3). The basal expression of *OsNPR1* was reduced in homozygous *OsNPR1*-RNAi lines, in comparison to wild-type Gui99 plants (Figs. 2a and 3c), whereas four paralogs of *OsNPR1* remained unaffected (Fig. 2b), confirming the specific suppression of *OsNPR1* in those *OsNPR1*-RNAi lines.

To construct *OsNPR1*-overexpressing vector, the full-length genomic DNA of *OsNPR1,* from the start codon to 192 bp downstream of the stop codon, was placed under the control of the maize ubiquitin promoter in the pCAMBIA1301-UbiN vector (Fig. 1b), and was introduced into rice cultivars Gui99 and TP309 by *Agrobacterium*-mediated transformation. Hygromycin-resistant regenerated Gui99 and TP309 rice plants were obtained and tested as

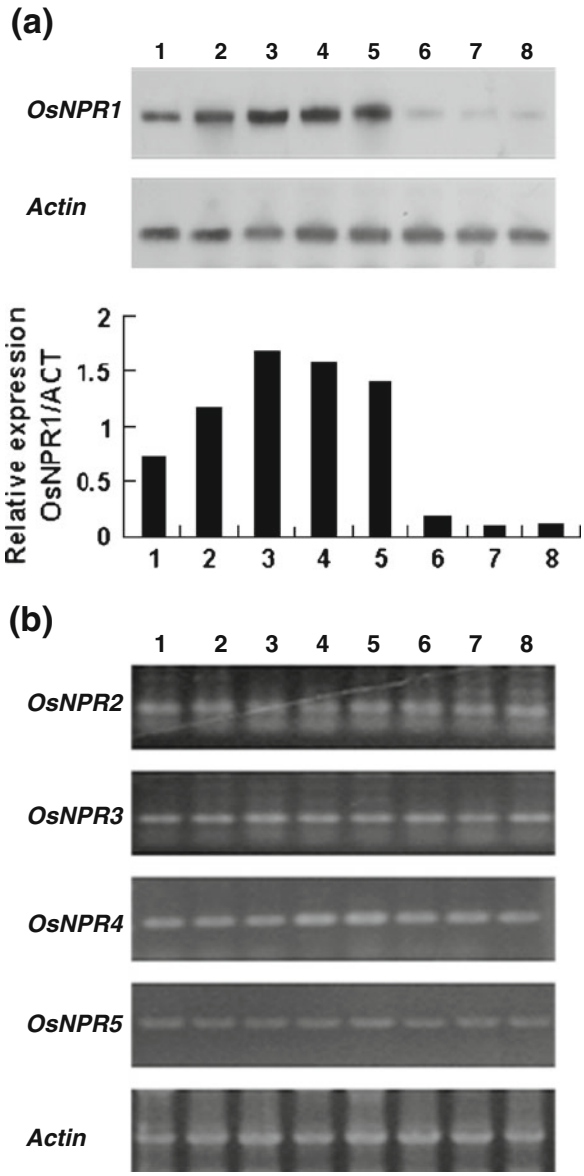

Fig. 2 Expression of *OsNPR1* and its paralogs in wild-type plant Gui99 and its transgenic lines. **a** Northern blot analysis of the expression of *OsNPR1* in wild-type plant Gui99 and its homozygous transgenic lines. The intensity of the hybridization bands was quantified using the Quantity One software (Bio-Rad, Munchen, Germany) and the relative expression of *OsNPR1* was normalized with *Actin*. 1, wild-type rice Gui99; 2–5, Gui99 *OsNPR1*-overexpressing lines 782, 783, 784, and 929; 6–8, Gui99 *OsNPR1*-RNAi lines I1, I3, and I6. **b** RT-PCR was performed using specific primers for the *OsNPR1* paralogs *OsNPR2*, *OsNPR3*, *OsNPR4*, *OsNPR5*. 1, wild-type rice Gui99; 2–5, Gui99 *OsNPR1*-overexpressing lines 782, 783, 784, and 929; 6–8, Gui99 *OsNPR1*-RNAi lines I1, I3, and I6

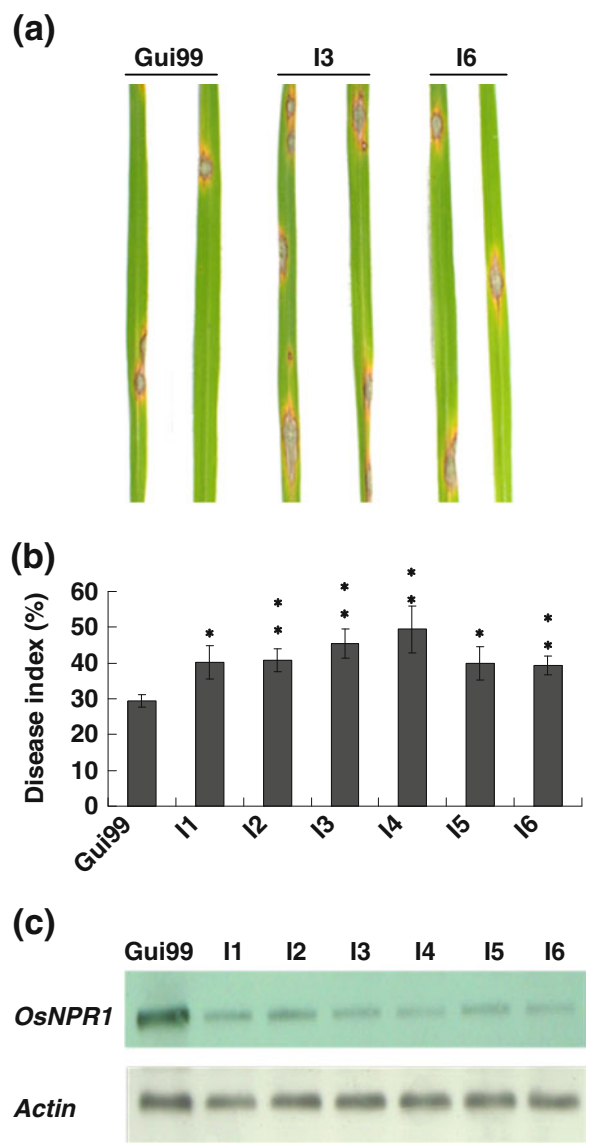

Fig. 3 The *OsNPR1*-RNAi plants are significantly more susceptible to rice blast fungus *M. oryzae*. **a** Lesion phenotypes of *OsNPR1*-RNAi homozygous plants after infection with *M. oryzae* strain CHL0742. Gui99 is untransformed control plant, while I3 and I6 are independent *OsNPR1*-RNAi transgenic lines. The photograph was taken 7 days post inoculation. **b** Average disease index of *OsNPR1*-RNAi homozygous plants after infection with *M. oryzae* strain CHL0742. The untransformed control plant Gui99 and six independent *OsNPR1*-RNAi transgenic lines (I1 to I6) were tested. Data are the means ± SD from three repeats, each using ten leaves. Statistically significant differences between transgenic plants and wild type plants are indicated by * ($P<0.05$) and ** ($P<0.01$). The experiments were repeated at least three times and similar results were obtained. **c** Northern blot analysis of the expression of *OsNPR1* in wild-type plant Gui99 and its *OsNPR1*-RNAi transgenic lines

transgenic plants by PCR amplification of the specific transgene or the hygromycin-resistant gene. Eight TP309 transgenic lines were randomly chosen for Southern hybridization. The results showed that the untransformed control plant TP309, and the eight transgenic lines, have a common hybridizing band representing the endogenous *OsNPR1* sequence; four out of the eight lines gave only one additional hybridizing band, indicating that *ca* 50% of the TP309 transgenic lines carried one copy of the integrated transgene (Figure S3). The basal expression of *OsNPR1* increased in homozygous Gui99 *OsNPR1*-overexpressing lines (Fig. 2a) and TP309 *OsNPR1*-overexpressing lines (Fig. 4e), in comparison to wild-type plants, whereas four paralogs of *OsNPR1* remained unaffected in Gui99-derived lines (Fig. 2b), demonstrating the enhanced expression of *OsNPR1* in those *OsNPR1*-overexpressing lines.

OsNPR1-RNAi plants were significantly more susceptible to rice blast fungus *M. oryzae* in addition to *X. oryzae* pv. *oryzae*

To investigate whether *OsNPR1* is involved in rice basal resistance to virulent *M. oryzae*, the homozygous *OsNPR1*-RNAi plants, together with the untransformed control plant Gui99, were inoculated with virulent *M. oryzae* strain CHL0742 in the greenhouse. In repeated tests, leaves of the wild-type rice Gui99 developed typical blast disease symptoms. The *OsNPR1*-RNAi plants showed larger lesions (Fig. 3a). Statistical analysis by the *t* test showed that the disease indices of the *OsNPR1*-RNAi lines were significantly higher than that of wild-type plant (Fig. 3b). These results demonstrate that *OsNPR1*is required for rice basal resistance to the blast fungus *M. oryzae*.

We also examined whether OsNPR1 plays a role in induced rice blast resistance by BTH. The results showed that BTH application significantly reduced the disease index of blast in wild-type plant Gui99 in comparison to mock treatment (Figure S4a,b). The disease indices of BTH-treated 2 lines of *OsNPR1*-RNAi plants were significantly higher than that of BTH-treated wild-type plant Gui99 (Figure S4a,b). These results demonstrate that OsNPR1 is involved in BTH-induced blast resistance. As shown in Figure S4c, BTH application enhanced the expression of *OsNPR1* in wild-type Gui99, whereas this effect was affected in the 2 lines of *OsNPR1*-RNAi plants.

In addition, the *OsNPR1*-RNAi plants were challenged by rice bacterial blight pathogen *Xoo* strain 13751. The result showed that suppression of *OsNPR1* caused rice plants to be more susceptible to *Xoo* (Figure S5).

Overexpression of *OsNPR1* in rice enhanced resistance to rice blast fungus *M. oryzae* in addition to *Xoo*

To further confirm the positive function of *OsNPR1* in basal resistance to *M. oryzae*, Gui99 *OsNPR1*-overexpressing lines, together with wild-type rice Gui99 were inoculated with *M. oryzae* strain CHL0742 in the greenhouse. The *OsNPR1*-overexpressing lines showed smaller lesions than wild-type Gui99 (Fig. 4a). Statistical analysis by the *t* test revealed that the disease indices on the tested Gui99 *OsNPR1*-overexpressing lines were significantly lower than those on Gui99 (Fig. 4b). Furthermore, the TP309 *OsNPR1*-overexpressing lines and its non-transgenic wild-type plant were also tested for blast resistance. Consistently, the disease indices on the tested *OsNPR1*-overexpressing lines (Fig. 4e) were significantly lower than those on control plant TP309 (Fig. 4c, d). These results indicated that overexpression of *OsNPR1* in either Gui99 or TP309 led to enhanced resistance to *M. oryzae* infection, further supporting the positive function of OsNPR1 in basal resistance to blast fungus.

As also shown in Figure S4, BTH-treated 2 lines of *OsNPR1*-overexpressing plants showed enhanced blast resistance and increased expression of *OsNPR1* in comparison to BTH-treated wild-type plant Gui99. In addition, *OsNPR1*-overexpressing lines were challenged with *Xoo* strain 13751. The transgenic plants were significantly more resistant to *Xoo* infection (Figure S6).

A set of defence genes was positively regulated by *OsNPR1* in rice basal resistance

The expression of rice defence genes *PR-1a*, *PBZ1, CHI*, *GLU* and *PAL*, as well as *OsNPR1*, was examined by Northern hybridization in randomly chosen *OsNPR1*-RNAi lines (I1 and I3), *OsNPR1*-overexpressing lines in Gui99 (782 and 784) and

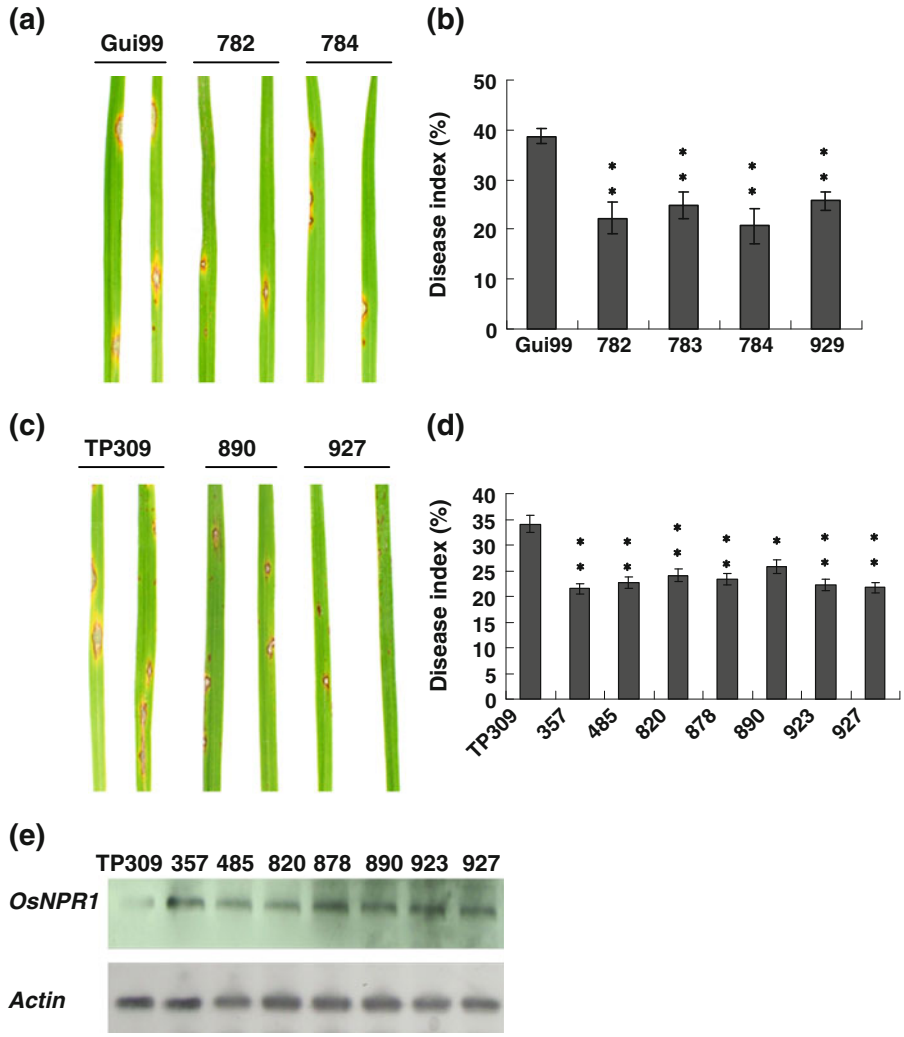

Fig. 4 *OsNPR1*-overexpressing plants showed enhanced resistance to rice blast fungus *M. oryzae*. **a** The symptom of *OsNPR1*-overexpressing lines in Gui99 caused by *M. oryzae* strain CHL0742. Gui99 is the wild-type plant; The numbers 782 and 784 represent independent Gui99 transgenic *OsNPR1*-overexpressing lines. The photograph was taken 7 days post inoculation. **b** Average disease index of *OsNPR1*-overexpressing lines in Gui99 after infection with *M. oryzae* strain CHL0742. Four Gui99 transgenic lines were tested. Gui99 was used as control. Data are the means ± SD from three repeats, each using 10 leaves. Statistically significant differences between transgenic plants and wild type plants are indicated by ** ($P<0.01$). The experiments were repeated at least three times and similar results were obtained. **c** The symptom of *OsNPR1*-overexpressing lines in TP309 caused by *M. oryzae* strain CHL0742. TP309 is the wild-type plant, 890 and 927 are independent TP309 transgenic plants. The photograph was taken 7 days post inoculation. **d** Average disease index of *OsNPR1*-overexpressing lines in TP309 after infection with *M. oryzae* strain CHL0742. Seven independent T3 TP309 transgenic *OsNPR1*-overexpressing lines were tested. TP309 was used as control. Data are the means ± SD from three repeats, each using 10 leaves. Statistically significant differences between transgenic plants and wild type plants are indicated by * ($P<0.05$) and ** ($P<0.01$). The experiments were repeated at least three times and similar results were obtained. **e** Northern blot analysis of the expression of *OsNPR1* in wild-type plant TP309 and its *OsNPR1*-overexpressing transgenic lines

TP309 (890 and 927) before and at different times after inoculation with *M. oryzae*, and compared with that of wild-type plants. First of all, the gene *OsNPR1* was induced in wild-type plants by *M. oryzae* infection as found by Yuan et al. (2007) (Fig. 5). Whereas in both *OsNPR1*-RNAi lines, the *OsNPR1* mRNA levels were lower than the endogenous level represented by the Gui99 wild-type control at

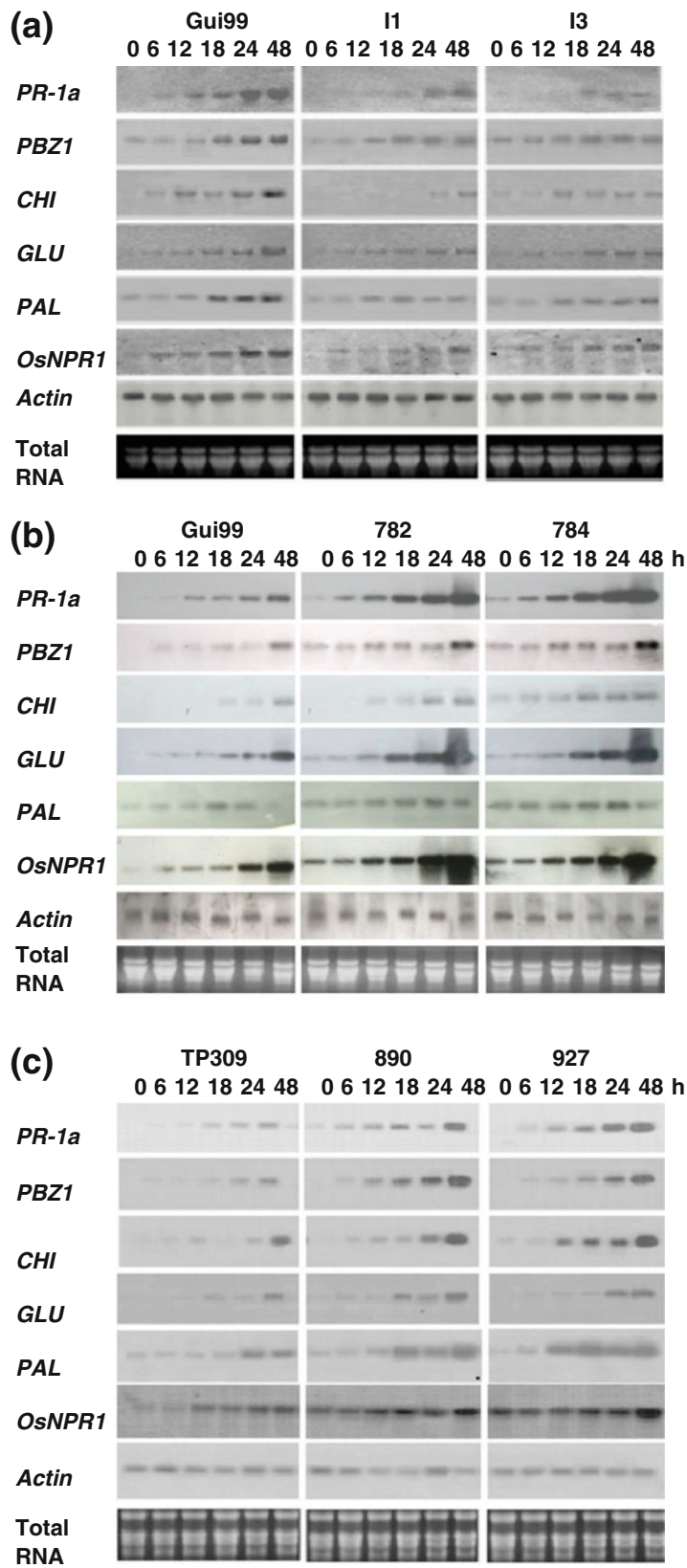

Fig. 5 Transcription levels of defence-related genes and *OsNPR1* in transgenic plants after inoculation with *M. oryzae*. Total RNAs were extracted from leaves 0 h, 6 h, 12 h, 18 h, 24 h, and 48 h after pathogen inoculation. RNA gel blots were hybridized with probes of *PR-1a*, *PBZ1*, *CHI*, *GLU*, *PAL*, *Actin*, and *OsNPR1* genes. **a** Decreased expression of defence-related genes in *OsNPR1*-RNAi lines after challenge with *M. oryzae* strain CHL0742. The lines I1 and I3 are independent *OsNPR1*-RNAi transgenic lines. Gui99 is the untransformed plant. **b**, **c** Increased expression of defence-related genes in *OsNPR1*-overexpressing lines after challenge with *M. oryzae* strain CHL0742. Gui99 and TP309 are wild-type plants. The numbers 782 and 784 represent two independent Gui99 transgenic *OsNPR1*-overexpressing lines. The numbers 890 and 927 represent two independent TP309 transgenic plants

different times after *M. oryzae* inoculation (Fig. 5a). In all *OsNPR1*-overexpressing lines, the *OsNPR1* transcripts were at higher levels than the endogenous level represented by the respective wild-type plant at different times post inoculation (Fig. 5b, c).

The five defence genes under study were induced in wild-type rice plants by *M. oryzae* infection (Fig. 5). Their expression levels increased gradually from 6 h to 48 h after inoculation (Fig. 5). In fungus-infected *OsNPR1*-RNAi plants, transcripts for all the five defence genes studied accumulated at lower levels than in fungal-infected wild-type plants at different time points after inoculation (Fig. 5a), indicating that the expression of the defence genes was suppressed in the *OsNPR1*-RNAi plants. But in fungus-infected *OsNPR1*-overexpressing plants, transcripts for all the five defence genes accumulated at higher levels than in fungal-infected respective wild-type plants (Fig. 5b, c), indicating that the expression of the defence genes was elevated in the *OsNPR1*-overexpressing plants. All these results demonstrate that *OsNPR1* positively regulates the expression of the five defence genes in the basal resistance of rice to the blast fungus *M. oryzae*.

Furthermore, the expression of defence genes *PR-1a*, *PAL* and *POD* was examined by Northern hybridization in the same set of transgenic rice lines as mentioned above before and after the plants were inoculated with *Xoo*, and compared with that of wild-type plants. As shown in Figure S7, the *OsNPR1* gene was induced in wild-type plants by *Xoo* infection as also found by Yuan et al. (2007). Whereas in all *OsNPR1*-overexpressing lines, the *OsNPR1* transcripts were at higher levels than the endogenous level represented by the respective wild-type plant post *Xoo* inoculation (Figure S7a, b). In both *OsNPR1*-RNAi lines, the *OsNPR1* mRNA levels were lower than the endogenous level represented by the wild-type control after *Xoo* infection (Figure S7 c). The three defence genes studied were induced in wild-type plants by *Xoo* infection (Figure S7). After *Xoo* inoculation, all the *OsNPR1*-overexpressing lines showed faster and stronger activation of the *PR-1a*, *PAL*, and *POD* genes in comparison with that in wild-type plants (Figure S7a, b). But the two independently generated *OsNPR1*-RNAi rice lines showed weaker activation of these defence genes post *Xoo* inoculation when compared with that in the wild-type rice (Figure S7). These results indicate that *OsNPR1* positively regulates the expression of the three defence genes in the basal resistance of rice to the bacterial pathogen *Xoo*.

Discussion

In this study, we cloned *OsNPR1/NH1* gene from the rice cultivar Gui99 and generated *OsNPR1*-RNAi rice lines in Gui99 and *OsNPR1*-overexpressing rice lines in cultivars Gui99 and TP309 with altered levels of only *OsNPR1* expression. The results of disease resistance assays in *OsNPR1*-RNAi rice plants and *OsNPR1*-overexpressing lines demonstrated that *OsNPR1* is involved in rice resistance to bacterial blight pathogen *Xoo* and BTH-induced resistance to blast fungal pathogen *M. oryzae,* which is consistent with the previous reports (Chern et al. 2005; Yuan et al. 2007; Shimono et al. 2007; Sugano et al. 2010) and also confirmed the validity of the plants used in this study. In contrast with previous reports which did not observe any effects of *OsNPR1* in rice basal resistance to the blast fungus *M. oryzae* (Yuan et al. 2007; Sugano et al. 2010), we provided direct evidence to show that *OsNPR1* is involved in basal resistance to *M. oryzae.* Differences in the genetic background of the rice cultivars and the blast fungal strains used, expression levels of transgene, rice growth conditions, and rice ages may explain these inconsistent results.

NPR1 has been actually found to play very important roles in basal resistance of *Arabidopsis*. The *Arabidopsis npr1* mutant was more susceptible to virulent *Pseudomonas syringae* pv. *maculicola* strain ES4326 than the wild-type plant in the absence of any pretreatments (Cao et al. 1994). In another independent study, Glazebrook et al. (1996) screened a mutagenized population of *Arabidopsis* for individuals that exhibited enhanced disease susceptibility (*eds*) to the moderately virulent bacterial strain *P. s.* pv. *maculicola* ES4326 and they found that two of the *eds* mutations were *npr1* alleles. Moreover, overexpression of NPR1 in *Arabidopsis* conferred enhanced disease resistance to both bacterial and fungal pathogens in the absence of any pretreatment (Cao et al. 1998; Friedrich et al. 2001).

Quilis et al. (2008) showed that expression of *Arabidopsis NPR1* in rice conferred resistance to *M. oryzae*, providing evidence for the possible involve-

ment of *OsNPR1* in rice basal resistance to the blast fungus. In the same study, they demonstrated that expression of *NPR1* in rice also conferred resistance to another fungal pathogen *Fusarium verticillioides* and bacterial pathogen *Erwinia chrysanthemi* (Quilis et al. 2008). Direct evidence for the involvement of *OsNPR1* in the rice basal resistance to *Xoo* comes from the work of Yuan et al. (2007). They demonstrated that lines of *OsNPR1*-RNAi in TP309 were more susceptible to *Xoo* compared with the wild-type plant and *OsNPR1*-overexpressing lines in TP309 showed enhanced resistance to *Xoo* (Yuan et al. 2007). Earlier studies also provided evidence for the involvement of *OsNPR1* in the basal resistance of rice to *Xoo*. Chern et al. (2005) showed that overexpression of *OsNPR1* in rice cultivar LiaoGeng conferred enhanced resistance to *Xoo* in the absence of any pretreatments. Notably, overexpression of *NPR1* in rice cultivar TP309 conferred enhanced resistance to *Xoo* in the absence of any pretreatments (Chern et al. 2001).

The defence genes *PR-1a*, *PBZ1, CHI*, *GLU*, *PAL* were chosen for checking their expression levels in transgenic plants and wild-type plants before and after *M. oryzae* inoculation. Our results showed a positive correlation between resistance levels of rice plants to the inoculated *M. oryzae* and expression levels of the five defence genes in rice, which may partially explain the phenotypes expressed by the transgenic rice lines altered in *OsNPR1* expression after *M. oryzae* inoculation. These results are also in line with previous report showing the correlation between the enhanced resistance of the rice lines overexpressing *Arabidopsis NPR1* gene to *M. oryzae* infection and faster and stronger activation of the defence genes *PR-1b*, *PBZ1*, *OsPR10* and *TLP* (Quilis et al. 2008).

A number of rice defence genes, such as *PR-1a*, *CHI*, *GLU*, and *PBZ1*, were found to be upregulated in rice leaves in responding to *M. oryzae* attack (Kim et al. 2004). Among the defence genes, *PR-1a* and *PBZ1* are the most well characterized genes in rice. The expression of *PR-1a* is often used as a marker for SAR development (Durrant and Dong 2004; Grant and Lamb 2006). PR-2 consists of 1,3-β-glucanases (GLU) and PR-3 contains various chitinases (CHI) which degrade fungal cell walls (Van Loon and Van Strien 1999). Plant GLUs may inhibit fungal pathogen growth directly (Kini et al. 2000). Constitutively expressing a rice *CHI* gene in transgenic rice plants led to increased disease resistance (Nishizawa et al. 1999). In addition, phenylalanine ammonia-lyase (PAL) has been proven to catalyze the first reaction in the biosynthesis of a wide variety of natural phenylpropanoid products, including lignin, flavonoids, pigments, and phytoalexins. It is a key enzyme in pathogen defence and the stress response (Van Loon and Van Strien 1999).

In this work, we only showed the positive regulation of the six selected defence genes by *OsNPR1* in rice basal resistance by Northern hybridization. It was reported that overexpression of *OsNPR1* in rice led to elevated expression of defence genes, *PR-1b*, *PBZ1*, *PAL*, and *POD* (Chern et al. 2005; Yuan et al. 2007) and the expression of *PR-1b* greatly decreased in *OsNPR1*-RNAi plants (Yuan et al. 2007). Microarray experiments showed that *NPR1* positively regulates the expression of *PR* genes and also the protein secretory pathway genes in *Arabidopsis* (Wang et al. 2005). Very recently, genome-wide transcript profiling was used to identify OsNPR1-dependent, BTH-responsive genes in rice (Sugano et al. 2010). It will be very interesting to know the genes regulated by OsNPR1 in rice basal resistance at the genome level.

In contrast with the previous reports (Fitzgerald et al. 2004; Chern et al. 2005; Quilis et al. 2008), we did not observe obvious developmental abnormalities including lesion-mimic phenotype in all our transgenic rice plants (data not shown). Yuan et al. (2007) also did not observe a reliable lesion-mimic phenotype on the leaves of all their transgenic *OsNPR1*-overexpressing lines. The differences in genetic background, expression levels of transgene, growth conditions perhaps can explain the discrepancies (Yuan et al. 2007), but more evidence is needed.

Rice blast disease is a serious agricultural problem and results in severe damage and reduces yields of planted rice. Therefore, disease resistance to this pathogen is of immense interest to rice breeders. In this study, we revealed a novel function for *OsNPR1* in positively regulating the rice basal resistance to *M. oryzae* and provided evidence for potential application of *OsNPR1* in engineering rice for broad spectrum disease resistance.

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