OsWRKY13 Mediates Rice Disease Resistance by Regulating Defense-Related Genes in Salicylate- and Jasmonate-Dependent Signaling

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Although 109 WRKY genes have been identified in the rice genome, the functions of most are unknown. Here, we show that OsWRKY13 plays a pivotal role in rice disease resistance. Overexpression of *OsWRKY13* can enhance rice resistance to bacterial blight and fungal blast, two of the most devastating diseases of rice worldwide, at both the seedling and adult stages, and shows no influence on the fertility. This overexpression was accompanied by the activation of salicylic acid (SA) synthesis-related genes and SA-responsive genes and the suppression of jasmonic acid (JA) synthesis-related genes and JA-responsive genes. OsWRKY13 bound to the promoters of its own and at least three other genes in SA- and JA-dependent signaling pathways. Its DNA-binding activity was influenced by pathogen infection. These results suggest that OsWRKY13, as an activator of the SA-dependent pathway and a suppressor of JA-dependent pathways, mediates rice resistance by directly or indirectly regulating the expression of a subset of genes acting both upstream and downstream of SA and JA. Furthermore, OsWRKY13 will provide a transgenic tool for engineering wider-spectrum and whole-growth-stage resistance rice in breeding programs.

Additional keyword: Oryza sativa.

Plants have evolved an efficient defense transduction network against pathogen attack. Although many components of this network are still unknown, the salicylic acid (SA)-dependent systemic acquired resistance (SAR) pathway and the jasmonic acid and ethylene (JA/ET)-induced resistance pathway are well-recognized (Glazebrook 2001; Hammond-Kosack and Parker 2003). SA, JA, and ET are each involved in the regulation of defense against different pathogens (Thomma et al. 1998). Resistance against biotrophic pathogens is usually regulated by the SA-dependent pathway, whereas resistance against necrotrophic pathogens and insects is usually controlled by the JA/ET-dependent pathway (Felton and Korth 2000). However, SA- and JA/ET-dependent defense signals interact with each other, either synergistically or antagonistically (Durrant and Dong 2004). Activation of SAR can suppress JA biosynthesis and JA-responsive gene expression, and SA deficiency can

result in accumulation of JA and enhanced expression of JA-responsive genes.

NPR1 is a regulatory protein in *Arabidopsis thaliana* that modulates pathogenesis-related (PR) gene expression, resulting in activation of SAR through binding to members of the TGA class of basic leucine-zipper transcription factor family. NPR1 is an important regulator in the antagonistic cross-talk between the two induced resistance pathways (Pieterse and Van Loon 2004; Zhang et al. 1999). Nuclear localization of NPR1 is required for SA-mediated defense gene expression (Kinkema et al. 2000), whereas suppression of JA signaling does not require nuclear localization of NPR1 (Spoel et al. 2003), indicating that NPR1 is not a direct regulator of JA-responsive gene expression.

Transcription activation of SA- and JA-responsive genes is essential for the induced resistance conferred by the two pathways. In addition to TGAs, WRKY transcription factors are also involved in modulation of SA- or JA-responsive gene expression and disease resistance (Asai et al. 2002; Chen and Chen 2002; Deslandes et al. 2002; Kim et al. 2006; Noutoshi et al. 2005; Robatzek and Somssich 2002; Xu et al. 2006; Yang et al. 1999; Zheng et al. 2006) in *Arabidopsis* and tobacco. WRKY70 is a node of convergence for integrating SA- and JA-mediated signals during *Arabidopsis* response to pathogens (Li et al. 2004). The WRKY proteins are a class of regulators that control diverse developmental and physiological processes in addition to disease-resistance responses (Eulgem et al. 2000). The rice WRKY superfamily consists of about 109 members, although the functions of most are unknown (Qu and Zhu 2006). Thus far, there has been no direct study of the role of rice WRKY protein in pathogen-induced defense regulation, although a few pathogen-induced WRKY genes have been identified (Liu et al. 2005b; Wen et al. 2003).

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* and fungal blast caused by *Magnaporthe grisea* are two of the most devastating diseases of rice worldwide. Our previous study showed that the cDNA clone EI1211, corresponding to *OsWRKY13* of the rice WRKY numbering system of Xie and associates (2005), increased expression after incompatible pathogen infection of several rice lines carrying different resistance (R) genes conferring resistance for *X. oryzae* pv. *oryzae* or *M. grisea*; no expression change of EI1211 was observed in compatible interactions (Wen et al. 2003). In addition, *OsWRKY13* (EI1211) colocalizes with a resistance quantitative trait locus against blast disease in rice chromosome 1 (Wen et al. 2003). Thus, we propose two hypotheses regarding the function of *OsWRKY13*. First, it may be involved in rice resis-

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tance in a pathogen nonspecific way, but activation of *R* genes is the key to initiate its function. Second, constitutive overexpression of *OsWRKY13* may enhance the defense of rice against diseases, regardless of the existence of an *R* gene.

To examine the hypotheses, we monitored *OsWRKY13* expression. Here, we show that overexpression of *OsWRKY13* in the genetic background without an *R* gene enhanced rice resistance to both *X. oryzae* pv. *oryzae* and *M. grisea*. In addition to phenotypic changes, *OsWRKY13*-overexpressing plants also showed expression profiling variation of a subset of defense-related genes, which were responsive to either JA, SA, or both, or were known to function in JA- or SA-dependent pathways. *OsWRKY13* showed different DNA-binding abilities before and after pathogen challenge. The expression characteristics of the defense-related genes in the transgenic plants and the interaction of *OsWRKY13*-DNA suggest that this protein mediates resistance by simultaneous direct or indirect activation of the genes in the SA-dependent pathway and suppression of the genes in the JA-dependent pathway.

RESULTS

OsWRKY13 modulates rice resistance to both bacterial blight and fungal blast.

Comparative analysis of the genomic and cDNA sequences of *OsWRKY13* (GenBank accession number EF143611) from

resistant cultivar Minghui 63 (*Oryza sativa* subsp. *indica*) showed that the gene was 1,505 bp in length and had a coding region interrupted by two introns (Supplemental Fig. 1A). The putative encoding product of *OsWRKY13* consisted of 316 amino acids, which contained a WRKY motif, a zinc-finger motif, and a potential nuclear localization signal (Fig. 1A). According to the classification of the WRKY superfamily (Eulgem et al. 2000), *OsWRKY13* belongs to group II.

OsWRKY13 was overexpressed in cultivar Mudanjiang 8 (*O. sativa* subsp. *japonica*). Minghui 63 was moderately resistant to *X. oryzae* pv. *oryzae* PXO61, although it carried two genes, *Xa25(t)* and *Xa26*, for bacterial blight resistance. Mudanjiang 8, without any known *R* gene, was highly susceptible to *X. oryzae* pv. *oryzae*. At the booting stage, 13 of the 47 plants showed significantly enhanced resistance to PXO61, with the lesion area ranging from 24.4 to 48.6% versus 61.8 and 32.2% for the controls of Mudanjiang 8 and Minghui 63, respectively. The enhanced resistance was stably inherited (Fig. 1B). The reduced lesion area of transgenic plants after *X. oryzae* pv. *oryzae* inoculation was associated with increased accumulation of *OsWRKY13* transcripts (Fig. 1C, Supplemental Table 1).

To verify that the resistance of the transgenic plants was due to overexpression of *OsWRKY13*, four T₂ families (D11UM1-4, D11UM7-2, D11UM18-9, D11UM18-10) from three resistant T₀ plants (D11UM1, D11UM7, D11UM18) were examined individually at the booting stage for resistance by inocula-

Fig. 1. Product and function of the *OsWRKY13* gene. **A**, Schematic diagrams of conserved motifs of *OsWRKY13*. NLS = nuclear localization signal, WRKY = WRKY motif, and zinc finger = zinc-finger motif. Conserved sequences of these structures are presented, and numbers indicate the amino acid position of each structure in *OsWRKY13*. **B**, *OsWRKY13*-overexpressing plants show enhanced resistance to *Xanthomonas oryzae* pv. *oryzae* PXO61 at both the seedling and booting stages. The T₂ transgenic plants (1-1, 7-2, 18-10) are from T₀ plants D11UM1, D11UM7, and D11UM18. 7-2N is a transgene-negative plant segregated in the D11UM7-2 family. **C**, Expression analysis of *OsWRKY13* in noninoculated T₀ transgenic plants, wild type, mock (water)-inoculated Minghui 63 (donor of *OsWRKY13*, M), and PXO61-inoculated Minghui 63 (P) by RNA gel blot analysis. **D**, Enhanced resistance to PXO61 cosegregates with overexpression of *OsWRKY13* in T₂ families D11UM1-4 and D11UM7-2. **E**, Growth of PXO61 in leaves of T₃ *OsWRKY13*-overexpressing (D11UM1) and wild-type plants. Bacterial populations were determined from three leaves at each timepoint by counting CFUs. Similar results were obtained in two independent biological experiments. **F**, Resistance of *OsWRKY13*-overexpressing plants for Blast. The T₁ negative (D11UM18-1N) and positive (D11UM18-4) plants were from T₀ plant D11UM18. Similar results were obtained in two independent biological experiments. ck = Mudanjiang 8 (wild type).

tion with PXO61 and measuring *OsWRKY13* expression level. The enhanced resistance cosegregated with the increased level of *OsWRKY13* transcripts in all T₂ families (Fig. 1D, result from only two families shown). The growth rate of bacteria on resistant transgenic plants was 5.5- to 13.9-fold lower than that on susceptible control Mudanjiang 8 at 2 to 14 days after inoculation (Fig. 1E). The resistance of *OsWRKY13*-overexpressing plants to PXO61 at the four- to five-leaf stage was also enhanced significantly, as evaluated by lesion area (Fig. 1B). These results suggest that overexpression of *OsWRKY13* can improve resistance against bacterial blight disease at both the seedling and adult stages.

Mudanjiang 8 was susceptible to *M. grisea*, which causes blast disease. After infection with *M. grisea* at the tillering stage in a natural infection field, the T₁ plants from *OsWRKY13*-overexpressing T₀ plant D11UM18 segregated into two groups, a highly susceptible group that had numerous lesions on the leaves and a partly resistant group that had few lesions on the leaves. The enhanced resistance of the plants cosegregated with both the overexpression of *OsWRKY13* and the expression of β -glucuronidase, which represented the presence of the transgene (Fig. 1F). The T₁ plants from T₀ plant D11UM47 were also examined for blast resistance by inoculation with *M. grisea* isolate V86013 in a greenhouse at the three- to four-leaf stage. Again, the enhanced resistance of T₁ plants cosegregated with overexpression of the *OsWRKY13* gene (data not shown). These results suggest that *OsWRKY13* was also involved in blast resistance at both the seedling and adult stages.

OsWRKY13 regulates the expression of other defense-related genes.

The expression patterns of two *PR* genes and eight genes known to function in SA- or JA-dependent defense signaling pathways in an *OsWRKY13*-overexpressing (D11UM7-2) line were examined. Six of the ten genes showed differential expression upon pathogen challenge in susceptible wild-type plant Mudanjiang 8. PXO61 challenge significantly induced the expression of *PR1a* (acidic PR protein, AJ278436), *LOX* (lipoxygenase, D14000), and *AOS2* (allene oxide synthase 2, AY062258) and suppressed the expression of *PR10* (ribonuclease, D38170), *PAL* (phenylalanine ammonia-lyase, X87946), and *CHS* (chalcone synthase, X89859) in Mudanjiang 8 (Fig. 2). The expression of *NHI* (*Arabidopsis* NPR1 homolog 1, AY923983) and *Arabidopsis* homologs *EDS1* (enhanced disease susceptibility 1, AK100117), *PAD4* (phytoalexin deficient 4, CX118864), and *ICS1* (isochlorismate synthase 1, AK120689) was not affected by pathogen inoculation in Mudanjiang 8.

OsWRKY13-overexpressing plants accumulated significantly more *PR1a*, *PAD4*, *ICS1*, and *CHS* transcripts and fewer *AOS2* and *PR10* transcripts than did the wild type (Fig. 2). Before pathogen infection, expression levels of *LOX* and *NHI* were significantly higher in *OsWRKY13*-overexpressing plants than in wild type. After inoculation, overexpressing *OsWRKY13* suppressed *LOX* and showed no significant influence on *NHI*, as compared with wild type (Fig. 2). The expression of *EDS1* and *PAL* was not significantly affected by the level of *OsWRKY13* transcript.

OsWRKY13 protein is targeted to the nucleus and binds to gene promoters.

To determine the subcellular localization of *OsWRKY13*, genomic DNA, and cDNA of *OsWRKY13* was fused in frame with the gene encoding green fluorescence protein (GFP). The *GFP-OsWRKY13* fusion gene driven by maize ubiquitin (*Ubi*) promoter was transiently expressed in onion epidermal cells. The recombinant fusion protein was localized in the nuclei (Fig. 3A to C), whereas cells bombarded with *Ubi:GFP* construct (control) yielded fluorescence in both cytoplasm and nuclei (Fig. 3D and E).

To determine if *OsWRKY13* had DNA-binding activity, a series of yeast one-hybrid assays were performed using DNA fragments harboring W boxes (TTGACC/T), W-like boxes, or both (TTGACA, TGACC/T, or TTGAC) from the promoter regions of *PR1a*, *AOS2*, *LOX*, *ICS1*, *NHI*, and *PAD4*. Detection of protein-DNA binding activity by growth performance on SD-Leu-Trp-His agar medium showed that *OsWRKY13* possessed specific DNA-binding ability, binding strongly to the promoters of *PR1a*, *AOS2*, and *LOX* and not to the promoters of *ICS1*, *NHI*, and *PAD4* (Fig. 3F and G).

The promoter of *OsWRKY13* contains a TTGAC W-like box. A gel mobility shift assay showed that nuclear proteins from *OsWRKY13*-overexpressing plants had stronger binding signal to the probe, harboring the W-like box, than that from wild type (Fig. 3H). Pathogen inoculation increased the DNA-binding activity of the nuclear proteins from wild-type plants and reduced the binding activity of the nuclear proteins from *OsWRKY13*-overexpressing plants (Fig. 3H). The glutathione-S-transferase (GST)-*OsWRKY13* fusion proteins but not GST also bound strongly to the DNA probe (Fig. 3I). The DNA-binding intensity of either *OsWRKY13* or nuclear proteins was reduced by the competition of unlabeled probe, indicating the specificity of the binding. The W-like box is partly overlapped with the binding site (TGACG) for TGA transcription factors in the DNA probe. To determine the binding specificity, the DNA probe was site-mutated (Fig. 3J). Both *OsWRKY13*

Fig. 2. Expression analysis of defense-related genes in *OsWRKY13*-overexpressing plants by quantitative reverse transcription-polymerase chain reaction. Samples were obtained before PXO61 inoculation (ck) and at 2 and 4 days postinoculation. The expression level of each gene in transgenic plants was calculated relative to that in noninoculated wild-type plants. Circles indicate a significant difference ($P < 0.05$) between noninoculated and inoculated plants and asterisks indicate a significant difference ($P < 0.05$) between the transgenic plant and corresponding wild type within the same treatment. The RNA sample for the transgenic plant was a mixture isolated from eight leaves of four *WRKY13*-overexpressing or wild-type plants. Similar results were obtained in two independent biological experiments.

and nuclear proteins showed similar binding intensity to the mutated probe 1, harboring a typical W box (TTGACC) and without TGA-binding site, as to the wild-type probe, but greatly reduced binding to the mutated probe 2, without W or W-like box and TGA-binding site (Fig. 3J). These results suggest that both *OsWRKY13* and the nuclear proteins bind specifically to the W-like box.

Fig. 3. Subcellular localization and DNA-binding activity of *OsWRKY13*. Photographs (A through E) of onion epidermal cells were taken under a confocal microscope. Scale bars = 100 μ m. **A**, Green fluorescent protein (GFP)-*OsWRKY13* expression in nuclei after incubation for 24 h. **B**, Propidium iodide-stained nuclei. **C**, Transmission photograph overlaying A and B. **D**, GFP (control) expression in cytoplasm and nucleus. **E**, Transmission photograph overlaying D. **F**, Yeast one-hybrid assay using *OsWRKY13* as target protein and target DNA (PR1a) with three W boxes from the promoter of *PR1a* and control DNA (W17) without a W box from the promoter of *OsWRKY13* as baits. Top, schematic distribution of yeast cells carrying different plasmids; 1, positive control (p53HIS2 plus pGAD-Rec2-53); 2, target DNA only; 3, *OsWRKY13* plus target DNA; 4, *OsWRKY13* plus control DNA (W17); 5, control DNA (W17) only. Middle, positive interaction between *OsWRKY13* and target DNA PR1a was verified in SD-Leu-Trp-His (SD-L-T-H) agar medium with 30 mM 3-amino-1, 2, 4-triazole. Bottom, yeast cells carrying different plasmids were grown for 2 days at 30°C in SD-Leu-Trp (SD-L-T) agar medium. **G**, Yeast one-hybrid assay using *OsWRKY13* as target protein and target DNA fragments from the promoters of *AOS2*, *LOX*, *ICS1*, *NH1*, and *PAD4* as baits. Positive control (p53HIS2 plus pGAD-Rec2-53); negative control (*OsWRKY13* only). **H**, Nuclear proteins bind to DNA harboring a W-like box by gel mobility shift assays. ck = without nuclear protein. Nuclear proteins were isolated from wild-type (Mudanjiang 8, WT) and *OsWRKY13*-overexpressing T₃ (D11UM1 and D11UM7, 1/7) plants at two days after PXO61-inoculation (PXO61) or before pathogen inoculation (Before). The “a” and “b” indicate 5- and 2.5- μ g nuclear proteins, respectively. The labeled probes with or without 50- or 100-fold ($\times$) unlabeled probes were used for the binding assay. **I**, *OsWRKY13* binds to DNA harboring a W-like box by gel mobility shift assays. Aliquots (0.5 μ g) of glutathione-S-transferase (GST) or GST-*OsWRKY13* were used for DNA binding. **J**, Mutation (m2) of W-like box (underlined) in the original DNA probe (w) influences protein binding in gel mobility shift assay. All of the binding assays were repeated at least twice with similar results.

OsWRKY13 influences the accumulation of SA.

Quantification of free SA and its conjugate, SA β -glucoside, showed that *OsWRKY13*-overexpressing plants accumulated more free SA than did wild-type plants, whereas wild-type plants had more SA β -glucoside (Fig. 4A).

Defense signal molecules influence the expression of *OsWRKY13*.

The universal defense signal molecules SA, its analogs thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), and 2,6-dichloroisonicotinic acid (INA), and JA and ethephon (an ethylene generator) all influenced the expression of *OsWRKY13* (Fig. 4B). BTH induced *OsWRKY13* expression at 5 min and its effect on *OsWRKY13* expression began to disappear at 60 min after treatment, whereas SA and INA continually activated *OsWRKY13* expression. The induced expression of *OsWRKY13* began at 5 min, and this effect began to disappear at 30 min after JA treatment. The activation of *OsWRKY13* stimulated by ethephon showed a similar pattern as that of BTH.

DISCUSSION

Several WRKY proteins are involved in disease resistance of dicot plants (Chen and Chen 2002; Journot-Catalino et al. 2006; Kim et al. 2006; Li et al. 2004; Robatzek and Somssich 2002; Yang et al. 1999; Zheng et al. 2006) and many WRKY genes have been identified in the rice genome (Liu et al. 2005b; Qu and Zhu 2006; Xie et al. 2005). However, *OsWRKY13* is the first WRKY protein characterized from rice that plays a pivotal role in disease resistance. Constitutive

Fig. 4. Salicylic acid (SA) level and the effect of signal molecules. **A**, *OsWRKY13* influences the accumulation of free SA and conjugated SA levels in the leaves of Mudanjiang 8 (ck) and *OsWRKY13*-overexpressing T₂ plants, D11UM1-1, D11UM1-4, and D11UM7-2. Asterisks indicate a significant difference ($P < 0.05$) between transgenic and wild-type plants. Each sample was from four to eight plants. Similar results were obtained in two independent biological experiments. FW = fresh weight. **B**, Defense signal molecules influence *OsWRKY13* expression in Minghui 63. The control (ck) plants were treated with water. Each sample contained ten leaf segments and was collected before treatment (0) or after treatment with thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), 2,6-dichloroisonicotinic acid (INA), jasmonic acid (JA), salicylic acid (SA), or ethephon (an ethylene generator). Similar results were obtained in two independent biological experiments.

expression of *OsWRKY13* can enhance rice resistance to bacterial blight and fungal blast at both the seedling and adult stages and influences the expression of defense-related genes in SA- and JA-dependent signaling. Thus, characterization of *OsWRKY13* gives a crucial view to the regulation of pathogen-induced defense transduction pathways in rice.

***OsWRKY13* regulates a subset of defense-related genes in different signaling transduction pathways.**

The WRKY superfamily regulates gene expression by binding to the W or W-like box (Eulgem et al. 2000). The nuclear localization and DNA-binding ability of *OsWRKY13* implies its role in the regulation of gene expression. The differential expression of eight of the ten defense-related genes in the SA- or JA-dependent pathways in *OsWRKY13*-overexpressing plants further suggest the control of gene expression by *OsWRKY13*. Sequence analysis demonstrated that these eight defense-related genes and *OsWRKY13* were clustered with W or W-like boxes in their promoter regions (Supplemental Fig. 2). Binding of *OsWRKY13* to the promoter regions of at least four genes (*OsWRKY13*, *LOX*, *AOS2*, *PR1a*) was confirmed. Thus, resistance conferred by *OsWRKY13* may be activated by its regulation of some defense-related genes through direct binding to their *cis*-elements.

We compared the whole-genome expression profiles of *OsWRKY13*-overexpressing (two independent lines) and wild-type plants using Affymetrix rice genome arrays (D. Qiu and S. Wong, *unpublished data*). A total of 26 transcription factors exhibited differential expression in *OsWRKY13*-overexpressing plants, based on the analysis with an estimated false discovery rate of <5% and a cutoff value of twofold change (Hu and Ma 2006; Tusher et al. 2001). This result suggests that the differential expression of some defense-related genes in *OsWRKY13*-mediated resistance may also be through the action of other transcription factors regulated by *OsWRKY13*.

OsWRKY13 and nuclear proteins from *OsWRKY13*-overexpressing plants bind specifically to the W or W-like box (Fig.

Fig. 5. Defense signaling model for the roles of *OsWRKY13* in regulation of salicylic acid (SA)- and jasmonic acid (JA)-dependent pathways. Thick solid lines indicate direct action of *OsWRKY13* has been confirmed by protein-DNA binding assays; thin solid lines, regulation may be executed by direct or indirect action; dotted lines, regulation has been confirmed in rice or other plant species; the line ending with arrow, activation; and the line ending with perpendicular short line indicates suppression.

3J). Nuclear proteins from *OsWRKY13*-overexpressing plants demonstrated stronger DNA-binding activity than that from wild type in the absence of pathogen challenge (Fig. 3H). No WRKY genes (except *OsWRKY13*) or TGA genes in *OsWRKY13*-overexpressing plants showed enhanced expression based on rice whole-genome array analysis (D. Qiu and S. Wong, *unpublished data*). These results suggest that the DNA-binding activity of nuclear proteins may be mainly due to the action of *OsWRKY13*. Not all the eight genes under the control of *OsWRKY13* showed similar expression patterns in *OsWRKY13*-overexpressing plants before and after pathogen challenge. For example, the expression level of *LOX* was significantly higher before bacterial infection and lower after bacterial infection in *OsWRKY13*-overexpressing plants than that in wild type. Pathogen challenge influenced the DNA-binding activity of *OsWRKY13*. These results suggest that *OsWRKY13* may have two functional conformations. The conformational change is associated with pathogen invasion that may modify the phosphorylation status of *OsWRKY13*. Previous studies have also reported that activation of certain WRKY transcription factors by phosphorylation or other modification is required for successful defense (Asai et al. 2002; Turck et al. 2004; Yang et al. 1999).

Some of the defense-related genes under direct or indirect control of *OsWRKY13* were apparently also regulated by one or more other factors in *OsWRKY13*-mediated resistance. For example, although the expression of *LOX* and *AOS2* was reduced in *OsWRKY13*-overexpressing plants as compared with that of wild type upon pathogen infection, the relative expression of these two genes increased significantly as compared with uninfected *OsWRKY13*-overexpressing plants (Fig. 2). Overexpression of *NH1* enhanced the expression of *PR10* (Chern et al. 2005), but the expression pattern of *NH1* in *OsWRKY13*-overexpressing plant was not the same as that of *PR10*. These results indicate that one or more other factors independent of *OsWRKY13* may also regulate some of the defense-related genes. Furthermore, the differential expression of some of the defense-related genes in the transgenic plants could be influenced by the non-true *in vivo* binding of *OsWRKY13* to the W or W-like box of their promoters due to the high nonphysiological concentration of *OsWRKY13* in the cells. Thus, comparative analysis of the expression of these genes using an *OsWRKY13*-loss of function mutant will decipher the physiological regulatory function of *OsWRKY13*.

***OsWRKY13* is involved in the regulation of both SA- and JA-dependent pathways.**

NPR1 is a modulator for the antagonistic interaction of SA- and JA-mediated signals in the disease resistance of *Arabidopsis* (Pieterse and Van Loon 2004), and it is a key regulator of SAR in *Arabidopsis* (Durrant and Dong 2004). NPR1 functions downstream of SA by regulating the expression of SA-responsive genes. The functional position of NPR1 in the JA-dependent pathway is unclear, but it is not directly involved in the regulation of JA-responsive gene expression. The *Arabidopsis* WRKY protein WRKY70 was identified as a common component in the SA- and JA-dependent pathways (Li et al. 2004), regulating the antagonistic interaction of the two defense transduction pathways. WRKY70 is downstream of NPR1 and activates SA-responsive genes; it is also downstream of JA and suppresses JA-responsive genes. Overexpression of NPR1 or rice NPR1 homolog NH1 can enhance rice resistance to *X. oryzae* pv. *oryzae*; in addition, NPR1 and NH1 interact with rice TGA proteins, suggesting that rice and *Arabidopsis* share a conserved signal transduction pathway controlling NPR1-mediated resistance (Chern et al. 2001,

2005). However, it is unclear whether NPR1 or NH1 also regulate the interaction of SA- and JA-mediated signals in rice disease resistance. The present data indicate that OsWRKY13, functioning upstream of NH1, may be involved in the antagonistic interaction between SA- and JA-dependent pathways by regulating the expression of a subset of genes involved in synthesis of SA, JA, and PR proteins through multiple positive and negative feedback loops. A proposed functional model of OsWRKY13 in rice defense responses is shown in Figure 5.

Unlike *Arabidopsis WRKY70*, whose expression is activated by SA and suppressed by JA (Li et al. 2004), expression of *OsWRKY13* was induced by both SA and JA. *OsWRKY13* expression affected the transcript levels of PR genes and NH1. Thus, one action site of OsWRKY13 should be downstream of SA and JA and upstream of NH1 and PR proteins in the defense signaling network (Fig. 5). Both SA and JA can induce the expression of *PR1a* and *PR10* in rice (Agrawal et al. 2000, 2001). However, the expression patterns of these PR genes in transgenic plants (Fig. 2) clearly indicated that OsWRKY13 activates *PR1a* and suppresses *PR10*. *PR1* expression is the marker of SAR (Durrant and Dong 2004). *PR1a* showed the similar expression pattern as the genes, such as *PAD4* and *ICS1*, putatively functioning in SA-dependent pathway. Therefore, *PR1a* may function in the SA-dependent pathway, whereas *PR10* most likely functions in the JA-dependent pathway because its expression pattern is similar to those of *LOX* and *AOS2* in JA signaling in OsWRKY13-mediated resistance (Figs. 2 and 5).

SA can be synthesized via the isochlorismate pathway and the phenylpropanoid pathway (Fig. 5) (Lee et al. 1995; Mauch et al. 2001). The isochlorismate pathway appears to be the main source of SA synthesis during SAR, and the phenylpropanoid pathway appears to be responsible for the rapid production of SA associated with local cell death in dicot plants (Durrant and Dong 2004; Wildermuth et al. 2001). *ICS1* is a key enzyme for SA synthesis in the isochlorismate pathway. Two other proteins required for some R gene-mediated resistance, *PAD4* and *EDS1*, function in a positive feedback loop to regulate SA synthesis upstream of *ICS1* in dicot plants (Falk et al. 1999; Feys et al. 2001; Jirage et al. 1999). Although no study has examined the involvement of *EDS1/PAD4* in the isochlorismate pathway in rice, evidence supports the existence of an SA synthesis pathway during SAR in rice similar to that in dicot plants. First, enhanced expression of *PAD4* and *ICS1* was accompanied by an increased level of free SA in *OsWRKY13*-overexpressing plants. Second, incompatible interaction induced *PAD4* expression, implying its role in R gene-mediated resistance (D. Qiu and S. Wong, unpublished data). *CHS* functions on a branch of the phenylpropanoid pathway and catalyzes synthesis of defensive secondary metabolites, such as phytoalexins, using the intermediate of SA synthesis as a matrix (Gozzo 2003). Overexpressing *OsWRKY13* also enhanced the expression of *CHS*. These results suggest that other action sites of OsWRKY13 in the SA-dependent pathway are upstream of *PAD4* and *CHS* and activate the synthesis of SA and phytoalexins, respectively, through a feedback control loop.

The JA synthesis pathway begins with α -linolenic acid, and *LOX* and *AOS* are two important enzymes in this pathway (Fig. 5) (Zhao et al. 2005). *AOS2* is important in accumulation of JA in rice (Mei et al. 2006). In *OsWRKY13*-overexpressing plants, the expression of *LOX* and *AOS2* was suppressed accompanying the activation of genes functioning in the SA-dependent pathway upon pathogen infection, indicating that OsWRKY13 may suppress the JA-dependent pathway by negative feedback regulation of JA synthesis.

Conclusion.

Some components involved in the cross-talk between SA- and JA-dependent defense pathways against pathogens and

insects have been revealed in dicot plants, but how this interaction is regulated in rice, which has a high basal SA level, is unknown. The present results show that OsWRKY13 can directly or indirectly regulate the expression of genes functioning upstream and downstream of SA and JA, providing a starting point for studying the interaction between SA- and JA-dependent pathways during rice disease resistance. Overexpression of *OsWRKY13* can enhance rice resistance to both bacterial and fungal pathogens without causing deleterious fertile effects. Thus, this protein also provides a transgenic tool for engineering wider-spectrum and whole-growth-stage resistance rice in breeding programs.

MATERIALS AND METHODS

Gene isolation and structural analysis.

EI1211 is the cDNA fragment of the *OsWRKY13* gene. A genomic bacterial artificial chromosome (BAC) library constructed with Minghui 63 tissues was screened using EI1211 as probe, and a positive BAC clone, 7G7, was identified. A fragment about 3.8 kb in length and harboring *OsWRKY13* was obtained from 7G7 by digestion with restriction enzyme *HindIII* and was subcloned into vector pUC19, and the subclone (7G7H14) was sequenced. The structure of *OsWRKY13* was determined by sequencing the transcript of the gene. The 3' untranslated region was analyzed by 3' rapid amplification of cDNA end (RACE) assays using the 3' Full RACE core set (TaKaRa Biotechnology Co., Ltd., Dalian, China), according to the manufacture's protocols, and *OsWRKY13*-specific primer WRKY4 (5'-GGATGGATCGACCAGTACCA-3'). The 5' untranslated region was determined by reverse transcription-polymerase chain reaction (RT-PCR), using primers WRKY14F (5'-CTGCGCTTAGTTTGTGGGTGGG-3') and WRKY6R (5'-GGAGGTGATCATCAGGAG-3'), designed according to the full-length cDNA of the *OsWRKY13* allele in rice cultivar Nipponbare (GenBank accession number AK067329).

Plant transformation.

The overexpression construct of *OsWRKY13* was made by inserting a 3-kb DNA fragment digested with *BamHI* from clone 7G7H14 into vector pU1301, which was modified by insertion of a maize ubiquitin promoter and a napoline synthase polyadenylation signal (NOS) terminator into the multiple cloning sites of vector pCambia1301. *Agrobacterium*-mediated transformation was performed according to the protocol of Lin and Zhang (2005).

Pathogen inoculation.

To evaluate bacterial blight disease, plants were inoculated with *X. oryzae* pv. *oryzae* PXO61 at the seedling or booting stage by the leaf-clipping method (Kauffman et al. 1973). Disease was scored by measuring the percent lesion area (lesion length/leaf length) at 2 to 3 weeks after inoculation. For blast disease evaluation, seedlings at the three- to four-leaf stage were inoculated with *M. grisea* isolate V86013 by the spraying method (Chen et al. 2001) or plants were infected in a natural infection nursery. Mock-inoculated (control) plants were treated under the same conditions, except that pathogen suspension was replaced with water.

RNA gel blot and quantitative RT-PCR analyses.

Aliquots (20 μ g) of total RNA were used for RNA gel blot analysis (Zhou et al. 2002). The cDNA fragment (EI1211) of *OsWRKY13* was used as a hybridization probe. RT-PCR was conducted as described by Wen and associates (2003). Quantitative PCR was performed, using the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) accord-

ing to the manufacturer's instruction. The PCR primers for all the genes are listed in Supplemental Table 2. The expression level of actin was used to standardize the RNA sample for each RT-PCR. The quantitative PCR reaction was in a 25- μ l volume containing 1 μ l of diluted reverse transcription product, 12.5 μ l of 2 $\times$ SYBR green PCR master mix (Applied Biosystems), and 0.32 μ M of each primer.

Subcellular localization of OsWRKY13.

OsWRKY13 was fused with *GFP* gene. The coding region was obtained by PCR amplification of the genomic DNA and the cDNA of *OsWRKY13*. The PCR products were cloned into vector pU1300. Transient expression of the fusion gene in white onion (*Allium cepa*) epidermal cells was performed, essentially as described by Liu and associates (2005a). The onion epidermal cells then were stained with propidium iodide (10 μ g/ml) for nuclei and were observed with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems, Heidelberg GmbH, Germany).

Yeast one-hybrid assays.

The interaction of OsWRKY13 protein with W or W-like box in a promoter region (Supplemental Table 3) was examined using a yeast one-hybrid assay according to the manufacturer's protocol (Clontech Yeast Protocols Handbook, BD Biosciences Clontech, Mountain View, CA, U.S.A.). The DNA fragment (W17) without a W or W-like box from the promoter region of *OsWRKY13* was used as control. Positive interactions were verified by growing on SD-Leu-Trp-His agar medium.

Gel mobility shift assay.

Rice leaves were harvested from six-leaf stage rice for isolation of nuclear extract, essentially using the method reported by Marzabal and associates (1998). For recombinant proteins, the full-length cDNA of *OsWRKY13* was cloned into pDEST15 to generate *GST-OsWRKY13* through a GATEWAY system (Invitrogen life technologies, Carlsbad, CA, U.S.A.). The fusion construct was then introduced into BL21-Codon-Plus(DE3)-RP *Escherichia coli* (Stratagene, La Jolla, CA, U.S.A.). The GST-OsWRKY13 fusion protein was purified using MagneGST glutathione particles (Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol. Gel mobility shift assay was applied as described previously (Urao et al. 1993). The synthetic DNA probe harboring a W-like box from the promoter of *OsWRKY13* was generated by mixing the two complementary oligonucleotides (TTTTATTGACGTTCTAGA and TCTAGAACGTCAAATAAAA).

Quantification of SA.

Each sample was harvested from four to eight plants at the booting stage. The SA samples were extracted with slight modification of the procedure of Bowling and associates (1994). In brief, leaves were ground under liquid nitrogen. Three replicates of each ground sample weighing 300 to 1,000 mg (exact weights were recorded) were placed in 3 ml of 80% methanol supplemented with 1 μ g of naphthaleneacetic acid standard as internal control and were left to extract at 4°C for 24 h in the dark. The average recovery rate of the procedure was approximately 75%. The organic extracts containing the free SA were analyzed by high-performance liquid chromatography (Agilent 1100; Agilent Technologies, Palo Alto, CA, U.S.A.) with an Agilent C18 Zorbax ODS column (250 $\times$ 5 mm) run at 25°C in 50% water (0.1% H₃PO₄) and 50% methanol with a flow rate of 0.6 ml/min. SA was detected using a 230-nm wavelength. The aqueous phase containing the conjugated SA was acidified with HCl to pH 1 and was boiled for 30

min to release SA from any acid-labile conjugated forms. The released free SA was then extracted with the organic mixture and was analyzed.

Chemical treatments.

Chemical treatments were applied according to the methods of Agrawal and associates (2000). Minghui 63 was grown in a greenhouse at 25°C, 70% relative humidity, and a 12-h photoperiod for 21 days. Approximately 2-cm long leaf segments were cut from the fully expanded leaves and were floated on 30 ml of solution containing the test compound in covered sterile petri dishes. Leaf segments wounded by cutting and floated on deionized water served as control. The experiments were repeated twice. SA, JA, ethephon, BTH, and INA were purchased from Sigma (St. Louis). The concentration of working solutions was 100 μ M.

Sequence analysis.

A 1.5-kb sequence upstream of the coding region of each defense-related gene was analyzed to identify the *cis*-regulatory elements W or W-like box using the computer program PLACE Signal Scan (a database of plant *cis*-acting regulatory DNA elements). The W and W-like boxes were classified according to the published criteria (Eulgem et al. 2000; Maleck et al. 2000).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

PLACE Signal Scan database:
www.dna.affrc.go.jp/PLACE/signalscan.html