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OsWRKY62 is a Negative Regulator of Basal and *Xa21*-Mediated Defense against *Xanthomonas oryzae* pv. *oryzae* in Rice

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ABSTRACT The rice *Xa21* gene, which confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), encodes a receptor-like kinase. Few components involved in transducing the *Xa21*-mediated defense response have yet been identified. Here, we report that XA21 binds to a WRKY transcription factor, called OsWRKY62. The *OsWRKY62* gene encodes two splice variants (*OsWRKY62.1* and *OsWRKY62.2*). OsWRKY62.1:smGFP2 and OsWRKY62.2:smGFP2 fusion proteins partially localize to the nucleus. Transgenic plants overexpressing *OsWRKY62.1* are compromised in basal defense and *Xa21*-mediated resistance to *Xoo*. Furthermore, overexpression of *OsWRKY62.1* suppresses the activation of defense-related genes. These results imply that OsWRKY62 functions as a negative regulator of innate immunity in rice, and serves as a critical mediator of both basal and race-specific defense responses.

Key words: rice; *OsWRKY62*; *Xa21*; defense response.

INTRODUCTION

Innate immunity provides the first line of defense against attack from a variety of pathogens. It is mediated by pathogen recognition receptors (PRRs), which activate defense signaling pathways upon perception of pathogens (Akira and Takeda, 2004). PRRs and components involved in the signaling cascades appear to share remarkable similarities in plants, insects, and mammals. In mammals, recognition of pathogens at the cell surface is mostly carried out by the Toll-like receptors (TLRs), which contain an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 (*Drosophila* Toll and human IL-1) receptor domain (TIR) (Werling and Jungi, 2003). The TIR domains commonly associate with the IRAK/Pelle family of serine/threonine cytoplasmic kinases.

In plants, a major group of PRRs that recognize pathogens at the cell surface are the receptor-like kinases (RLKs), which contain various putative extracellular ligand-binding domains and an intracellular kinase domain related to the IRAK/Pelle kinase family (e.g. XA21 and FLS2) (Song et al., 1995; Shiu and Bleecker, 2001; Gomez-Gomez and Boller, 2002). Receptor-like proteins (RLPs) that lack intracellular kinase domains (e.g. XA21D and CF9) are hypothesized to dimerize with RLKs to transduce the resistance response (Jones et al., 1994; Wang et al., 1998). RLKs comprise one of the largest gene families in plants, with more than 600 members in the *Arabidopsis* genome and more than 1100 members encoded in rice genomes (Shiu and Bleecker, 2001; Shiu et al., 2004; Dardick and Ronald, 2006; Morillo and Tax, 2006). To date, all identified plant RLKs that function as PRRs fall into the non-RD class of kinases defined by the absence of an arginine-aspartic acid (RD) motif in the catalytic region (Dardick and Ronald, 2006). These include rice XA21, XA26, and Pi-d2 (Song et al., 1995; Sun et al., 2004; Chen et al., 2006), *Arabidopsis* FLS2, PR5K, and EFR (Wang et al., 1996; Gomez-Gomez and Boller, 2002; Zipfel et al., 2006), soybean RHG1 (Ruben et al., 2006; Afzal and Lightfoot, 2007), and wheat LRK10 and TaRLK-R1, 2, and 3 (Feuillet et al., 1997; Zhou et al., 2007). Extensive efforts have been devoted to understanding FLS2-mediated innate immunity; however, the non-RD RLK-mediated defense response in monocots remains to be elucidated.

A key mechanism of PRR-triggered innate immunity is the activation of defense-related genes, as mediated by

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transcription factors (Chisholm et al., 2006). For example, in mammals, upon pathogen recognition, TLRs interact with IRAK via adaptors like MyD88, which triggers mitogen-activated protein kinase (MAPK) cascades and subsequently leads to the activation of transcription factor NF-κB and the expression of immune response genes (Nurnberger et al., 2004). In plants, recent studies have shown that WRKY transcription factors are key regulators of the plant immune response (Eulgem and Somssich, 2007). For example, in *Arabidopsis*, WRKY transcription factors 22 and 29 function downstream of FLS2. Overexpression of the *WRKY29* gene constitutively activates the plant defense response against bacteria (Asai et al., 2002). Also in *Arabidopsis*, loss of *AtWRKY70* function compromises both basal defense responses to bacterial and fungal pathogens and *RPP4*-mediated race-specific resistance to *Hyaloperonospora parasitica* (Li et al., 2004, 2006; Knoth et al., 2007). In barley, overexpression of either *HvWRKY1* or *HvWRKY2* compromises both the basal defense response and MLA10-mediated race-specific resistance to *Blumeria graminis* (Shen et al., 2007).

WRKY proteins comprise a superfamily of mostly plant-specific transcription factors. A common feature of WRKY proteins is the WRKY domain that is defined by a region of approximately 60 amino acids containing a conserved WRKY amino acid sequence adjacent to a zinc-finger-like motif (Eulgem et al., 2000). Both regions are necessary for high-affinity binding to a canonical W-box motif containing a consensus sequence (C/T)TGAC(C/T). The W-box motif is over-represented in the promoter regions of several defense-related genes (Eulgem et al., 2000; Yu et al., 2001; Dong et al., 2003; Zipfel et al., 2004; Yamasaki et al., 2005; Shimono et al., 2007).

Although over 80 WRKY gene family members have been identified in the rice genome (Xie et al., 2005), the functional role of only a few rice WRKY factors has been explored in any detail. For instance, transgenic rice plants overexpressing *OsWRKY03* accumulate elevated defense-related gene transcripts relative to non-transgenic plants (Liu et al., 2005). Overexpression of *OsWRKY71* and *OsWRKY13* enhances resistance to *Xoo* in rice (Liu et al., 2006; Qiu et al., 2007), whereas, plants with reduced amounts of *OsWRKY45* expression exhibit compromised resistance to blast disease (Shimono et al., 2007). Despite these intriguing results, the upstream regulators of rice WRKY factors in defense signaling pathways have not yet been identified, and no WRKY factor has been shown to function directly downstream of a rice non-RD RLKs.

Rice *Xa21* confers gene-for-gene resistance to *Xoo* strains carrying AvrXa21 activity (Song et al., 1995; Lee et al., 2006). AvrXa21 is a type I secreted molecule that is predicted to bind XA21 and XA21D at the cell surface (Wang et al., 1998; da Silva et al., 2004; Lee et al., 2006). *Xa21* codes for a non-RD RLK with an extracellular LRR domain, a juxtamembrane (JM) domain, and an intracellular serine/threonine kinase domain (EC 2.1.7.). Studies on RLKs have indicated that the JM region of RLKs can serve as a binding site for downstream signaling proteins (Pawson, 2004). To identify potential components of the *Xa21*-mediated defense response, we employed a yeast two-hybrid system to screen for proteins that interact with XA21. A rice cDNA clone encoding OsWRKY62 was isolated. Here, we report the functional characterization of *OsWRKY62* in the rice immune response. The *OsWRKY62* gene encodes two splice variants: *OsWRKY62.1* and *OsWRKY62.2*. Overexpression of *OsWRKY62.1* compromises both basal defense and *Xa21*-mediated resistance to *Xoo*. Moreover, defense-related gene expression in *OsWRKY62.1*-overexpressing rice plants is suppressed. These data support a role for OsWRKY62 as a negative regulator of plant innate immunity.

RESULTS

Isolation of OsWRKY62

A truncated XA21 (XA21K668), which carries the JM region and the serine–threonine kinase domain of XA21, was used as a bait in a yeast two-hybrid screen to identify XA21 binding partners (Figure 1A). Of 7×10^7 transformants screened, eight classes of cDNA clones were isolated. One cDNA class encodes a WRKY transcription factor OsWRKY62, the longest clones of which appear to cover the complete open reading frame. To assess whether the JM region of XA21 is required for its interaction with OsWRKY62, XA21K(TDG) (Figure 1A) lacking the XA21 JM domain was co-expressed with OsWRKY62 in yeast. No interaction was observed between OsWRKY62 and XA21K(TDG). Furthermore, OsWRKY62 did not interact with a kinase-deficient mutant XA21 (XA21K736E) in which the invariant lysine in the catalytic domain was mutated to glutamic acid (Figure 1A). These data suggest that the XA21 and OsWRKY62 interaction requires the JM region and an active kinase domain of XA21.

According to the gene annotation in the J. Craig Venter Institute database (www.tigr.org), the *OsWRKY62* gene possesses two alternative splicing forms, named *OsWRKY62.1* and *OsWRKY62.2* (Figure 1B). The deduced amino acid sequence of OsWRKY62.1 is the same as that of the clone identified from the yeast two-hybrid analysis. In addition to a conserved WRKY domain, OsWRKY62.1 carries a putative sumoylation site at the C-terminus and a potential coiled-coil domain at the N-terminus. In contrast, OsWRKY62.2 only contains a small fraction of the N-terminal coiled-coil domain (Figure 1C).

RT–PCR analyses were performed to examine whether both forms are expressed in rice leaves using the primers indicated in Figure 1B. Amplified products were confirmed by sequencing. Figure 1D shows that both splice forms are expressed in plants and that expression of *OsWRKY62.1* dominates.

OsWRKY62 Belongs to the WRKY IIa Subfamily

OsWRKY62 is placed into group IIa of the WRKY superfamily according to the phylogenetic analyses of amino acid sequences of the predicted WRKY proteins in the sequenced rice

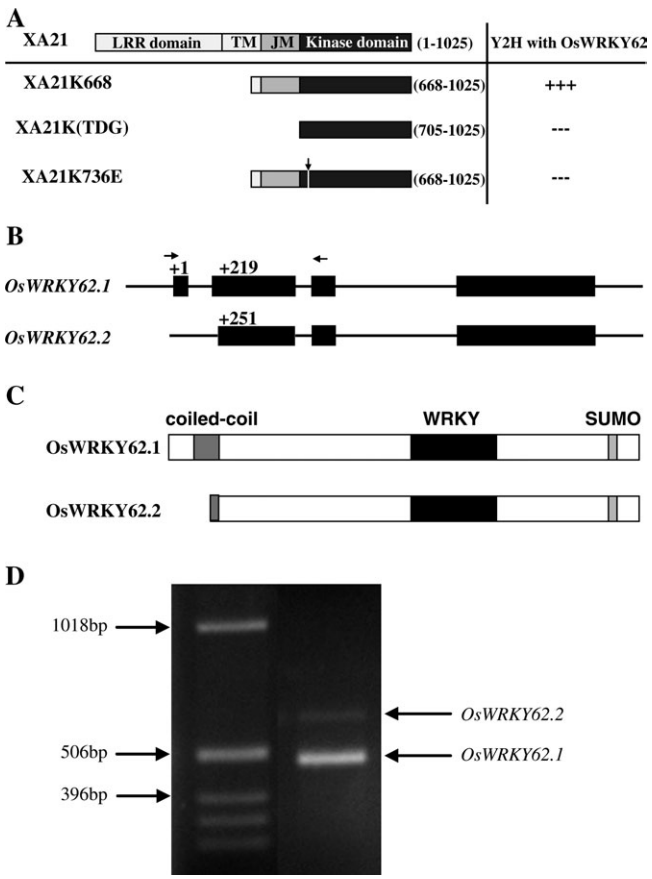

Figure 1. Protein Interaction of OsWRKY62 with XA21 in Yeast.

(A) Schematic diagram of XA21K668, XA21K(TDG), and XA21K736E used in the yeast two-hybrid analysis. TM indicates the transmembrane domain. JM indicates the juxtamembrane domain. Domains are as previously described (Song et al., 1995). The location of the conserved lysine (K736) in the kinase domain is indicated by an arrow. +++ indicates a strong interaction --- indicates no interaction.

(B) Schematic representation of the genomic structures of *OsWRKY62.1* and *OsWRKY62.2*. The exons and introns are represented by boxes and lines, respectively. The numbering of the nucleotides is based on the translation start site in *OsWRKY62.1* (numbered +1). Positions of primers used in the RT–PCR analysis are indicated by arrows.

(C) Schematic diagram of OsWRKY62.1 and OsWRKY62.2 protein structures. Boxes represent positions of domains including: the coiled-coil domain, the WRKY domain, and the sumoylation site (SUMO).

(D) RT–PCR analysis of *OsWRKY62.1* and *OsWRKY62.2* mRNA accumulation in rice leaves.

genomes (Eulgem et al., 2000; Xie et al., 2005). The phylogenetic relationships among WRKY IIa subgroup members from different organisms were further analyzed by comparing the protein sequences of the conserved WRKY domains. The *Arabidopsis* WRKY22 and WRKY29, which represent the WRKY IIe subfamily, were defined as outgroups. As shown in Figure 2, OsWRKY62 and OsWRKY76 mapped in the same subclade, suggesting a recent duplication within the clade. The comparison of the WRKY domain sequences indicates that OsWRKY62 is

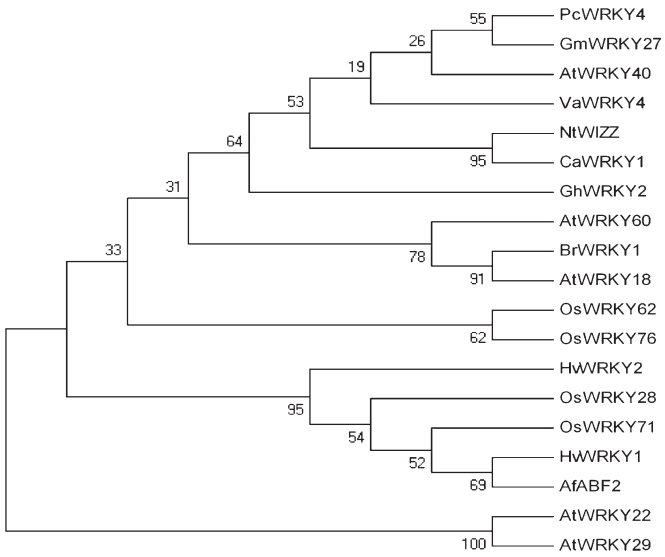

Figure 2. Phylogenetic Relationships between Plant WRKY IIa Subfamily Members.

Amino acid sequences of the conserved WRKY domains were aligned by ClustalW and then used in the molecular evolutionary genetics analysis (Mega). Numbers along branches are bootstrap percentage values. AtWRKY22 and AtWRKY29 were defined as outgroups.

90% identical to OsWRKY76. The other two rice WRKY IIa members (OsWRKY28 and OsWRKY71) were placed in the same subclade with HvWRKY1, HvWRKY2, and AfABF2.

Subcellular Localization of OsWRYK62.1 and OsWRKY62.2

To determine the subcellular localization of OsWRKY62.1 and OsWRKY62.2, OsWRKY62.1:smGFP2 and OsWRKY62.2:smGFP2 constructs were generated by fusing each cDNA lacking the termination codon to the coding region of the soluble-modified green fluorescent protein (smGFP2) (Davis and Vierstra, 1998). The maize ubiquitin promoter was used to drive the expression of the fusion proteins (Christensen and Quail, 1996). Each construct was delivered into rice protoplasts by PEG-mediated transformation (Abel and Theologis, 1994; Bart et al., 2006). The NLS:RFP construct, which contains a classical nuclear localization sequence (NLS), was co-expressed as a nuclear marker. Figure 3 shows that both OsWRKY62.1 and OsWRKY62.2 were detected in two locations. In a significant fraction of cells, the majority of the fusion proteins localized to an unidentified structure or structures, usually located adjacent to or overlapping with the nucleus, while a small portion localized to the nucleus. In other cells, the fusion proteins located to a structure or structures usually found near the nucleus, but nuclear signal was less obvious (not shown).

OsWRKY62 Is a Negative Regulator of Basal Defense against *Xoo* in Rice

To elucidate the putative function of OsWRKY62 in basal defense response to pathogens, *OsWRKY62.1* was constitutively expressed in rice plants under the control of the maize

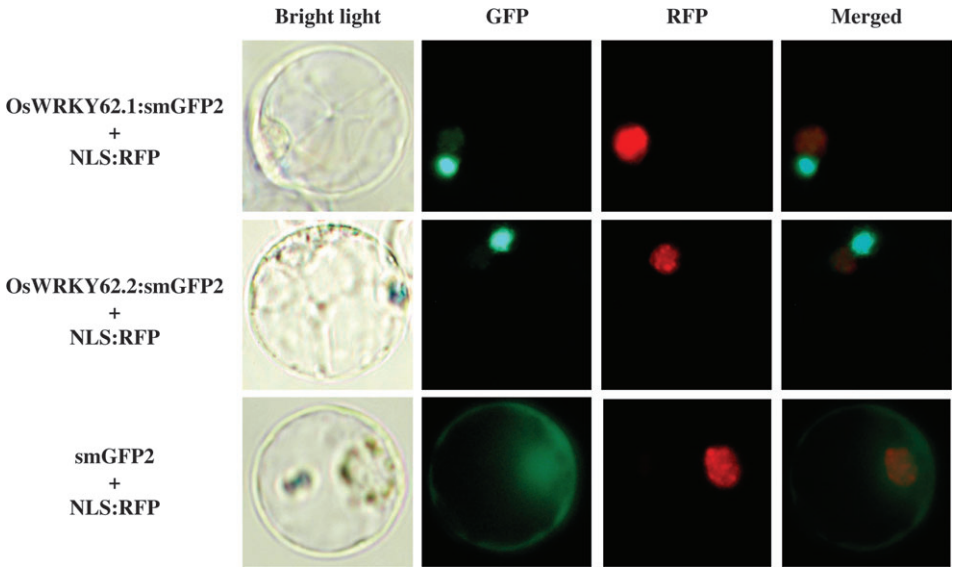

Figure 3. Subcellular Localization Patterns of OsWRKY62.1 and OsWRKY62.2.

The expression of OsWRKY62.1:smGFP2 and OsWRKY62.2:smGFP2 fusion proteins was driven by the maize ubiquitin promoter. As a control, an smGFP2 empty vector was transformed into separate cells. This experiment was repeated three times with similar results.

ubiquitin promoter. The *OsWRKY62.1* overexpression construct (OsWRKY62.1ox) was introduced into the rice cultivar, 'Kitaake', by *Agrobacterium*-mediated transformation. Twenty-five independently transformed lines were generated and challenged with *Xoo* at 5 weeks of age. Thirteen lines developed significantly longer lesions than the wild-type Kitaake controls (data not shown). No morphological changes were observed in these transgenic plants compared to the wild-type plants.

To verify whether the enhanced susceptibility phenotype is due to overexpression of *OsWRKY62.1* and not a positional effect of the transgene, the T1 progeny from three OsWRKY62.1ox lines (lines 3, 8, and 16) were further characterized. The presence of the OsWRKY62.1ox transgene in each plant was first examined by PCR. Amounts of *OsWRKY62.1* mRNA in the leaves of transgenic plants were monitored by reverse transcription–PCR (RT–PCR). Figure 4A shows that *OsWRKY62.1* mRNA accumulated to a higher degree in plants carrying the OsWRKY62.1ox transgene compared to the Kitaake control. The T1 progeny containing the OsWRKY62.1ox transgene were then subjected to *Xoo* inoculation at 5 weeks of age. Transgenic plants overexpressing *OsWRKY62.1* exhibited longer lesions compared to the wild-type controls (Figure 4B). Figure 4C shows two typical leaves from OsWRKY62.1ox lines 8 and 16 and the Kitaake controls. Bacterial growth curve analysis confirmed that a larger *Xoo* population accompanied the long-lesion phenotype in OsWRKY62.1ox plants (Figure 4D). These data support the hypothesis that the enhanced susceptibility phenotype in transgenic plants is caused by overexpression of *OsWRKY62.1*.

Plants overexpressing *OsWRKY62.2* were also generated and inoculated with *Xoo*. No clear effects on lesion development were observed for the *OsWRKY62.2* overexpressing plants (data not shown).

The Activation of Defense-Related Genes Is Suppressed in OsWRKY62.1ox Plants

To elucidate the molecular basis of the negative effect of *OsWRKY62.1* on rice defense response to *Xoo*, we carried out RT–PCR analysis to compare the expression of defense-related genes in the OsWRKY62.1ox lines and the Kitaake control. Total leaf RNA was extracted from OsWRKY62.1ox lines 8 and 16 as well as Kitaake plants immediately prior to inoculation (0 d) and 4 d after *Xoo* inoculation. Four defense-related genes (*Betv1*, *PR1a*, *PR10*, and *PBZ1*) were examined using gene-specific primers. Figure 5 indicates that at 4 d after *Xoo* inoculation, expression of the monitored genes was significantly induced in the wild-type plants. By contrast, accumulation of these genes was strongly suppressed in the OsWRKY62.1ox lines. These results suggest that constitutive expression of *OsWRKY62.1* suppresses defense-related gene expression, which likely contributes to the enhanced susceptibility phenotype in the OsWRKY62.1ox plants.

Overexpression of *OsWRKY62.1* Compromises *Xa21*-Mediated Resistance to *Xoo*

Because OsWRKY62 interacts with XA21 in yeast and blocks the activation of defense-related genes in rice, we reasoned that OsWRKY62 may negatively regulate the *Xa21*-mediated defense response to *Xoo*. To test this hypothesis, a transgenic rice line carrying the *Xa21* gene under its native promoter was generated in Kitaake rice using a mannose selectable marker (Kit-Xa21). Transgenic Kit-Xa21 plants were fully resistant to *Xoo*. We then introduced the OsWRKY62.1ox construct into

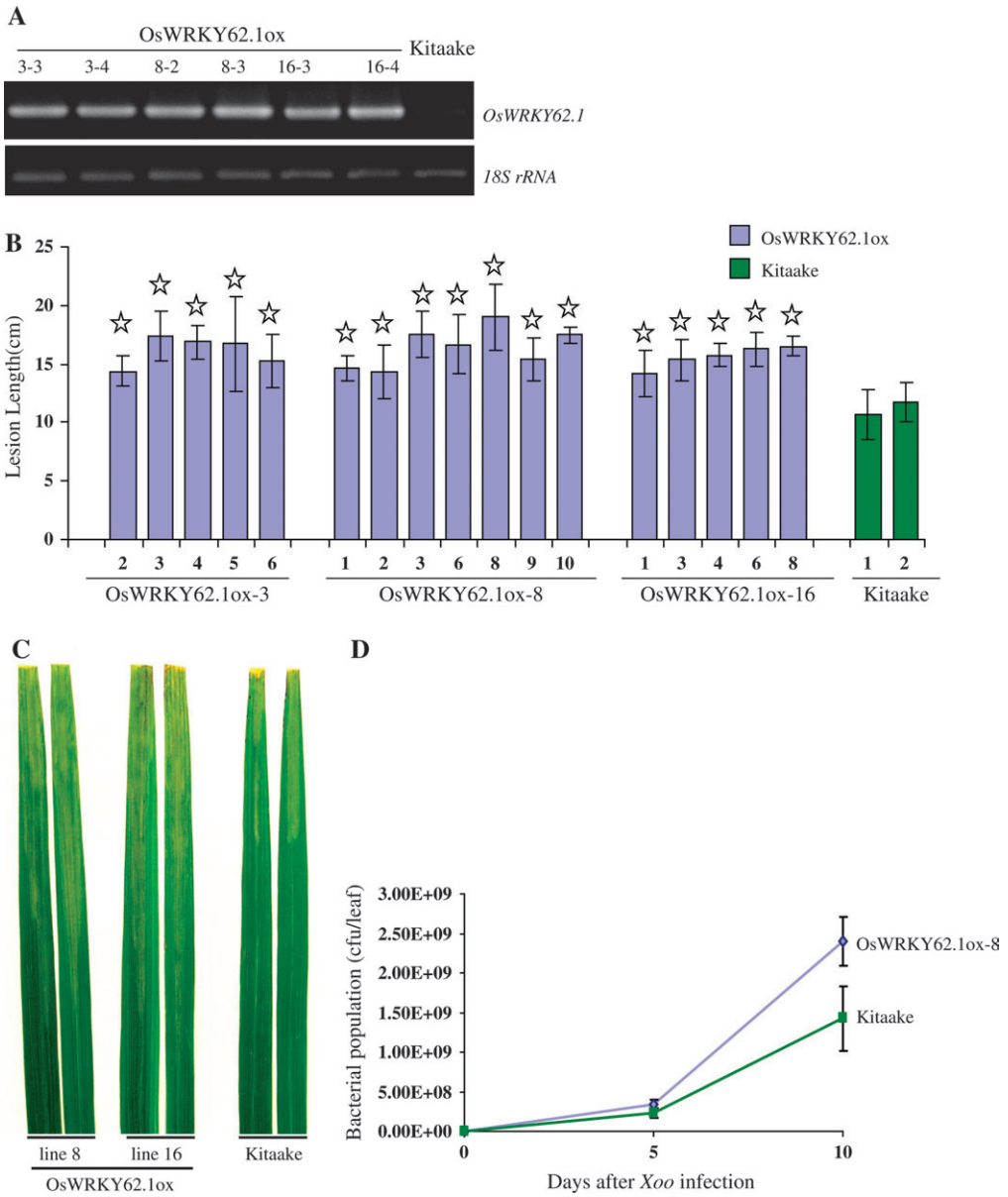

Figure 4. *OsWRKY62.1* mRNA Amounts and *Xoo* Inoculation Results for OsWRKY62.1ox Transgenic Plants.

(A) Expression of *OsWRKY62.1* mRNA in T1 progeny of OsWRKY62.1ox lines 3, 8, and 16 and the Kitaake control. RT–PCR was performed using *OsWRKY62.1*-specific primers. 18S ribosomal RNA was used as an internal control.

(B) Leaf lesion lengths of T1 progeny carrying the OsWRKY62.1ox transgene challenged with *Xoo*. Leaves were inoculated when the plants were 5 weeks old; lesion lengths were measured 10 d afterwards. Each bar represents the average and standard deviation of at least three leaves.

(C) Water-soaked lesions on leaves of T2 progeny of OsWRKY62.1ox lines 8 and 16 and the Kitaake control. The picture was taken 9 d after *Xoo* inoculation.

(D) *Xoo* population in T1 progeny of OsWRKY62.1ox line 8 and the Kitaake control. For each time point, the bacterial population size was determined separately for three leaves of similar growth stage from three plants. Error bars represent the standard deviation of three leaves. Stars indicate a significant difference ($P < 0.05$) between transgenic plants and wild-type plants according to t-test analysis.

a homozygous Kit-Xa21 line using a hygromycin selectable marker. Among 12 independently transformed plants, three lines (Xa21-OsWRKY62.1ox-1, –2, and –6) were identified as having highly increased accumulation of *OsWRKY62.1* mRNA by RT–PCR (Figure 6A) and characterized in detail. These three lines were challenged with *Xoo* at 5 weeks of age. All of them exhibited longer lesion lengths compared to the Kit-Xa21 controls (data not shown).

The T1 progeny of Xa21-OsWRKY62.1ox lines 2 and 6 were further characterized to check co-segregation between the

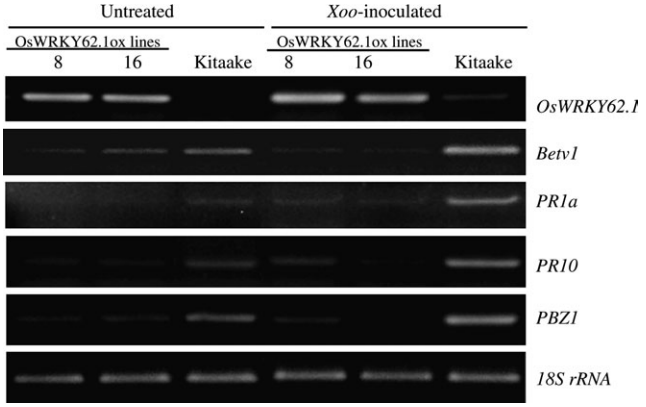

Figure 5. Effects of *OsWRKY62.1* Overexpression on Accumulation of mRNA from Defense-Related Genes.

Leaf tissue was collected from 5-week-old rice plants, including the Kitaake control and OsWRKY62.1ox lines 8 and 16, immediately prior to treatment and 4 d after *Xoo* inoculation. Total RNA samples were extracted and subjected to RT–PCR analysis with gene-specific primers for *OsWRKY62.1*, *Betv1*, *PR1a*, *PR10*, and *PBZ1*. 18S ribosomal RNA was used as an internal control.

OsWRKY62.1ox transgene and the susceptible phenotype. We examined the presence of the OsWRKY62.1ox transgene by PCR and challenged the progeny with *Xoo*. Lesion lengths were measured 8 d after inoculation. As seen in Figure 6B, in both lines tested, progeny carrying the OsWRKY62.1ox transgene in the Kit-Xa21 background showed longer lesion lengths than the null segregants without the OsWRKY62.1ox transgene. Figure 6C shows typical leaf samples after *Xoo* infection from each line. The Xa21-OsWRKY62.1ox lines developed water-soaked lesions similar to those observed in Kitaake controls. By contrast, lesion development was restricted to the site of *Xoo* infection in Kit-Xa21 plants. To compare the *Xoo* population in Xa21-OsWRKY62.1ox plants with the Kit-Xa21 and Kitaake controls, we carried out *Xoo* growth curves. Figure 6D and 6E show that there is a correlation between the longer lesion length and the larger *Xoo* population in Xa21-OsWRKY62.1ox plants. Taken together, these data support the conclusion that overexpression of *OsWRKY62.1* compromises *Xa21*-mediated resistance to *Xoo*.

DISCUSSION

OsWRKY62 Functions as a Negative Regulator of Plant Innate Immunity

The rice resistance protein XA21 represents a large class of RLKs predicted to be involved in innate immunity. Yeast two-hybrid screening was employed to identify protein candidates that might be involved in *Xa21*-triggered signaling cascades. A WRKY transcription factor, OsWRKY62, was identified through this screen. OsWRKY62, together with OsWRKY28, OsWRKY71, and OsWRKY76, comprise the rice WRKY IIa subfamily. It has been previously reported that transcripts of *OsWRKY62* and *OsWRKY76* accumulate in response to benzothiadiazole (BTH), salicylic acid (SA), and the rice fungal pathogen *Magnaporthe grisea* (Ryu et al., 2006; Shimono et al., 2007). However, overexpression of *OsWRKY62* and *OsWRKY76* has no effect on resistance to a compatible race of *M. grisea* (Shimono et al., 2007). In contrast, overexpression of *OsWRKY71*, which is induced by SA and rice bacterial pathogen treatment, leads to partial resistance to a virulent race of *Xoo* (Liu et al., 2006). In this study, we provide evidence that OsWRKY62 functions as a negative regulator of plant basal defense and *Xa21*-mediated resistance against *Xoo* carrying AvrXa21 activity.

WRKY transcription factors generally function through W-boxes, which are enriched in the promoters of several defense-related genes (Maleck et al., 2000). Constitutive expression of *OsWRKY62.1* in Kitaake plants causes enhanced susceptibility to *Xoo*, which is accompanied by suppression of defense-related gene expression, suggesting that *OsWRYK62.1* negatively regulates plant defense responses by interfering with pathogen-induced defense gene activation. Repression of defense response genes may be due to direct binding of OsWRKY62.1 to the promoters of the examined genes, as OsWRKY62.1 itself possesses transcriptional repression activity (LEB and PCR, unpublished results). Recent studies on *Arabidopsis* WRKY IIa subfamily members have implicated *WRKY18*, *WRKY40*, and *WRKY60* in negatively regulating the basal defense response to *Pseudomonas syringae* (Xu et al., 2006b). *wrky18 wrky40* and *wrky18 wrky60* double mutants and the *wrky18 wrky40 wrky60* triple mutant accumulate more transcripts of the defense-related gene, *PR1*, and exhibit significantly enhanced resistance to *P. syringae*, relative to wild-type plants. In addition, HvWRKY1 and HvWRKY2, two WRKY IIa subfamily members from barley were shown to suppress basal defense against *B. graminis* (Shen et al., 2007). These observations suggest a conserved negative regulatory function of WRKY IIa subfamily members in both dicots and monocots.

It has been shown that *Arabidopsis* WRKY IIa members form both homocomplexes and heterocomplexes mediated by their N-terminal Leucine-zipper motifs—a type of coiled-coil domain (Xu et al., 2006b). Association of different WRKY IIa members may alter their sequence-specific DNA-binding activities. Similarly, a subset of TGA transcription factors is found to exhibit increased DNA-binding activity by interacting with *Arabidopsis* NPR1—a key protein regulating systemic required resistance (Despres et al., 2000). These studies suggest that the activity of transcription factors may be regulated by interacting with other regulatory partners.

OsWRKY62.1 contains a putative N-terminal coiled-coil domain and a putative C-terminal sumoylation site. The coiled-coil domain is a common protein–protein interaction motif. Sumoylation has also been found to be involved in a variety of biological processes such as modulation of protein–protein interactions, regulation of the activity of the transcription factors, and alteration of target sub-cellular localization and stability (Seeler and Dejean, 2003). It is possible that the

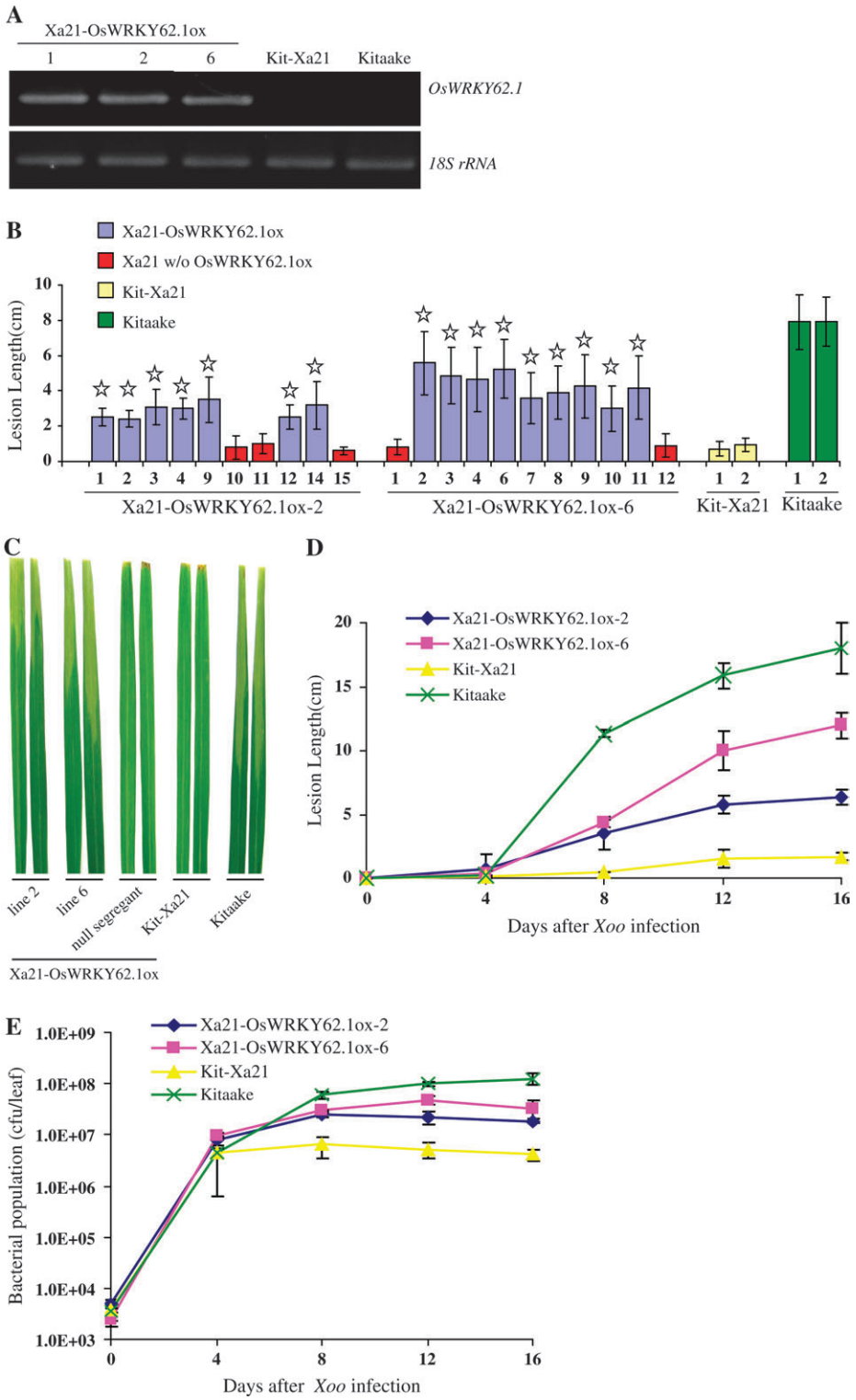

Figure 6. RNA Analysis and *Xoo* Inoculation Results of Kit-Xa21 Plants Overexpressing *OsWRKY62.1*.

(A) mRNA expression of *OsWRKY62.1* in the individually transformed Xa21-OsWRKY62.1ox lines 1, 2, and 6, Kit-Xa21 and Kitaake control plants. RT–PCR was performed using *OsWRKY62.1*-specific primers.18S ribosomal RNA was used as an internal control.

(B) Leaf lesion lengths of T1 progeny of Xa21-OsWRKY62.1ox lines 2 and 6, challenged by *Xoo*. Kit-Xa21 and Kitaake plants were used as controls. Leaves were inoculated when the plants were 5 weeks old; lesion lengths were measured 8 d afterwards. Each bar represents the average and standard deviation of at least five leaves. Stars indicate a significant difference ($P < 0.05$) between the transgenic plants containing the OsWRKY62.1ox transgene and null segregants according to t-test analysis.

putative coiled-coil domain and/or the putative sumoylation motif may serve as a docking site for the interaction of OsWRKY62.1 with other signaling components, which, in turn, regulates the function of OsWRKY62.1.

Constitutive expression of defense genes can be beneficial for plant disease resistance; however, it can also lead to aberrant regulation of cell death and deleterious effects on plant developmental processes, often causing morphological defects such as stunted growth and lower fertility (Lorrain et al., 2003; Fitzgerald et al., 2005). Furthermore, studies have demonstrated antagonistic relationships between pathways that function to defend against specific classes of pathogens, such as between the SA- and JA-mediated pathways (Glazebrook, 2005). Thus, the negative regulatory function of the *OsWRKY62* gene product inferred from our characterization of this gene suggests that this and other negative regulators of defense response may function as intrinsic controllers of plant innate immunity, which are critical for normal plant growth. In addition, negative regulation may function to fine tune defense responses to particular pathogens.

Functional Differences between OsWRKY62.1 and OsWRKY62.2

Alternative splicing is a major mechanism for generating mRNA and protein diversity. Alterations in protein-coding regions regulated by alternative splicing can lead to changes such as binding properties, sub-cellular localization, enzyme activities, and protein stability (Stamm et al., 2005). Analysis of rice cDNAs suggests that at least 16% of transcripts are alternatively spliced (Kikuchi et al., 2003). A number of WRKY genes in *Arabidopsis* and rice are predicted to have alternative open reading frames (Xie et al., 2005). However, to date, there have been no reports for the functional characterization of alternative splicing forms of WRKY genes.

The OsWRKY62.2 protein lacks a 39 amino acid segment from the N-terminus of OsWRKY62.1, which is predicted to contain a coiled-coil domain. *OsWRKY62.1* was predominantly expressed in rice leaves compared to *OsWRKY62.2*. Sub-cellular localization of these two forms was found to be similar. Interestingly, overexpression of *OsWRKY62.1* in rice plants enhanced susceptibility to *Xoo* pathogens, while no clear phenotypic change was observed for *OsWRKY62.2* overexpressing transgenic plants. These findings indicate that the N-terminal region of the OsWRKY62.1 protein may regulate OsWRKY62.1 function in defense response, possibly by interacting with other regulatory molecules, whereas OsWRKY62.2 may lack this ability.

Interaction between XA21 and OsWRKY62

The physical interaction between OsWRKY62 and XA21 was identified by the yeast two-hybrid method. This interaction is abolished when the XA21 JM domain is absent or the kinase catalytic activity of XA21 is impaired. In-vitro phosphorylation studies have shown that XA21 can autophosphorylate three residues—Ser686, Thr688, and Ser689—in the JM region (Xu et al., 2006a). The kinase-deficient XA21 mutant is unable to autophosphorylate (Liu et al., 2002). According to these observations, the yeast two-hybrid data suggest that the autophosphorylated XA21 JM region serves as a docking site for OsWRKY62. Similar mechanisms have been observed in other RLK-mediated signaling. For example, the type I transforming growth factor beta (TGF-β) receptor is activated by phosphorylation of the JM region. JM phosphorylation of the TGF-β receptor eliminates the binding site for the inhibitory protein FKBP12 and dramatically enhances specific binding with Smad2 (Huse et al., 2001).

Studies of *OsWRKY62* suggest a functional link between OsWRKY62 and XA21 in the plant defense response to *Xoo*. However, the location of in vivo interaction remains to be determined. In barley, recognition of Avr_A molecules by MLA10, an intracellular PRR, induces the association between HvWRKY2 and MLA10 in the nucleus, which interferes with the repressor function of HvWRKY2 in defense response (Shen et al., 2007). The *Arabidopsis* gene *RRS1-R* encodes an intracellular PRR which contains a WRKY domain at the C-terminus (Deslandes et al., 2003). It has been shown that nuclear localization of RRS1-R is dependent on the presence of its cognate Avr protein PopP2 (Deslandes et al., 2003). Furthermore, the WRKY domain of RRS1-R functions as a negative regulator of transcriptional activation of defense signaling (Noutoshi et al., 2005). According to the Rosetta Stone hypothesis, if two separate proteins in one organism can be found fused into a single protein in another organism, these two separate proteins may physically interact (Marcotte, 2000). The RRS1-R protein provides an intriguing example of a PRR that carries a WRKY factor. These observations suggest the existence of a mechanism in which PRRs directly bind to WRKY transcription factors and regulate the innate immune response. However, until now, there have been no reports of interaction of WRKY transcription factors with RLKs recognizing pathogen associated molecules present at the cell surface.

According to the observation that overexpressed OsWRKY62 proteins localize to an unidentified structure(s) often adjacent to the nucleus and to a lesser extent to the nucleus, we propose two models for the physical interaction

(C) Water-soaked lesions on leaves from T1 progeny of Xa21-OsWRKY62.1ox lines 2 and 6, Kit-Xa21 and Kitaake controls taken at 8 d after *Xoo* inoculation.
(D) The lengths of the lesions on T1 progeny of Xa21-OsWRKY62.1ox lines 2 and 6, Kit-Xa21, and Kitaake plants measured at days 0, 4, 8, 12, and 16 after *Xoo* inoculation. Each data point represents the average and standard deviation of three leaves.
(E) *Xoo* growth curve analysis of T1 progeny of Xa21-OsWRKY62.1ox lines 2 and 6, Kit-Xa21, and Kitaake plants. For each time point, the bacterial population size was determined separately for three leaves of similar growth stage from three plants. Error bars represent the standard deviation of three leaves.

between XA21 and OsWRKY62 during defense response in plants. In model 1, upon extracellular recognition of the AvrXA21 pathogen-associated molecule, autophosphorylated XA21, either the full-length protein or a cleaved form, is translocated to the nucleus, where it associates with OsWRKY62 through the phosphorylated XA21 JM domain. In support of the hypothesis that the XA21 RLK itself moves to the nucleus, recent studies have shown that endocytosis of plasma membrane-localized receptors occurs in both animals and plants. In animals, studies on TLR4, a receptor for lipopolysaccharides, indicate that TLR4-mediated immune responses are compromised by inhibition of endocytosis of TLR4 (Husebye et al., 2006). Similarly, in plants, endocytic processes have been observed for the surface receptor FLS2, suggesting that ligand-induced internalization of FLS2 might be involved in the innate immune signaling (Robatzek et al., 2006). Another possibility is that XA21 is proteolytically cleaved within the JM domain, which releases the cytoplasmic region from the membrane (Xu et al., 2006a). It is reasonable to hypothesize that a truncated form of XA21 may move into the nucleus and directly associate with OsWRKY62. This situation has been observed for human ErbB4, a receptor tyrosine kinase that recognizes neuregulin 1. After binding with its ligand ErbB4 is cleaved by a protease, liberating the intracellular domain of ErbB4 (Rio et al., 2000; Ni et al., 2001). A complex that includes the liberated portion of ErbB4, the adaptor TAB2, and the corepressor N-CoR then translocates to the nucleus, where it represses gene expression (Sardi et al., 2006). These observations support the hypothesis that the intracellular region of XA21 may be released from the membrane by proteolytic cleavage and subsequently interact with OsWRKY62 in the nucleus.

The second class of models we propose is that upon recognition of AvrXa21, the autophosphorylated XA21 JM domain binds and phosphorylates OsWRKY62 in the cytoplasm. With or without other binding partners, phosphorylated OsWRKY62 translocates to the nucleus and regulates the expression of defense genes. Support for this model comes from studies in the TGF-β pathway (Massague and Chen, 2000). TGF-β ligands bind to type I and type II serine/threonine kinase receptors. Subsequently, the activated ligand–receptor complex directly binds to and phosphorylates receptor-regulated Smads. Once phosphorylated, these receptor-regulated Smads associated with common Smad proteins and cotranslocate to the nucleus, where Smads can either positively or negatively regulate gene expression by association with other transcription regulators. Future studies on colocalization and in-vivo interaction of XA21 and OsWRKY62 upon infection by *Xoo* will shed light on the mechanistic basis of XA21-mediated OsWRKY62 activities in plants.

To date, understanding of the mechanism that integrates different signals resulting from pathogen infection is limited. Rice NRR, an NH1 (the rice homolog of *Arabidopsis* NPR1) binding protein, is found to negatively regulate both basal and *Xa21*-mediated resistance to *Xoo* (Chern et al., 2005a). The study of *OsWRKY62* provides additional evidence for the existence of a mechanistic link between these two defense responses. Moreover, identification of OsWRKY62 as a key component in *Xa21*-triggered signaling pathways suggests that OsWRKY62 may transduce the defense response mediated by other non-RD RLKs. Further investigation on the possible function of OsWRKY62 downstream of XA26 and Pi-d2 will help understand the specificity of OsWRKY62 in non-RD RLKs-mediated immune response.

METHODS

Plant Materials and Growth Conditions

Rice (*Oryza sativa L.*) plants were grown in the greenhouse until 5 weeks of age (unless stated otherwise) and transferred to the growth chamber before *Xoo* inoculation. In the greenhouse, the light intensity in photosynthetic photon flux across the spectrum from 400 to 700 nm was approximately 250 μmol m^{-2} s^{-1} in spring. Relative humidity in the greenhouse was >80%. The growth chamber was set on a 14-h daytime period, a 28/26°C temperature cycle and at 90% humidity. The chamber was equipped with metal halide and incandescent lights. The light intensity in the growth chamber was approximately 100 μmol m^{-2} s^{-1}.

Xoo Inoculation and Determination of Bacterial Populations

For *Xoo* inoculation, a bacterial suspension (OD_{600} of 0.5) of *Xoo* strain PX099 (Philippine race 6, PR6), which carries ArvXA21 activity, was used to inoculate rice by the scissors-dip method (Kauffman et al., 1973).

To determine the bacterial populations, three inoculated leaves from each genotype were ground up and resuspended in 10 ml H_2O to harvest bacteria separately. Diluted extract was plated on peptone sucrose agar plates containing 15 mg L^{-1} cephalexin.

Plasmid Construction and Rice Transformation

To generate the construct Xa21/C4300 for transforming rice with *Xa21* with mannose selection, a 9.8-kb KpnI DNA fragment (Song et al., 1995) containing the *Xa21* coding sequence plus 2204-bp 5′ and 3787-bp 3′ of the gene was cloned into the KpnI site in the C4300 vector. The C4300 vector, containing a phosphomannose isomerase (PMI) gene for selection in rice, was created by replacing the hygromycin resistance gene in the pCambia 1300 vector with the *PMI* gene from *E. coli* using the XhoI site (Chern et al., 2005a).

A 957-bp cDNA fragment encoding the full-length OsWRKY62.1 protein was PCR amplified from the original yeast two-hybrid cDNA clone, C1, using primers 5′-CACCATGGACGACGACGGCGACGGCT-3′/5′-CTACAAATGAACAGGAATGTG-3′. A 840-bp cDNA fragment encoding the full-length OsWRKY62.2 was PCR amplified using primers 5′-CACCATGGAGGAGAACGCGCGGCT-3′/5′-CTACAAATGAACAGGAATGTG-3′. The PCR products were cloned into the pENTR™/D-TOPO vector and the sequences confirmed by sequencing. The full-length *OsWRKY62.1* and *OsWRKY62.2* cDNAs were then cloned into the Ubi-NC1300-Rfa vector using the Gateway® LR clonase

(Invitrogen, Carlsbad, CA, USA), yielding the *OsWRKY62.1* overexpression construct (OsWRKY62.1ox) and the *OsWRKY62.2* overexpression construct (OsWRKY62.2ox), respectively. The ubi-NC1300-Rfa vector was generated by adding an additional reading frame cassette A into the Ubi-NC1300 vector (Chern et al., 2005b) according to the Gateway® Vector Conversion System instruction manual (Invitrogen).

Rice transformation was as described previously (Chern et al., 2001). *Agrobacterium* EHA105 was used to infect rice callus. Mannose (Lucca et al., 2001) was used for selection when *Xa21* was transformed into the rice cultivar Kitaake. For transformation of Kitaake or Kitaake containing *Xa21* (Kit-Xa21), hygromycin was used for selection. Rice lines OsWRKY62.1ox and Xa21-OsWRKY62.1ox were generated by transforming the OsWRKY62.1ox construct into the Kitaake and the Kit-Xa21 plants, respectively.

Yeast Two-Hybrid Screening

To construct the Xa21K668 bait plasmid, Xa21K668 was PCR amplified with the primer pair 5′-GTCGACATCATCACTCTACTTGCTTAT-3′/5′-CTGCAGTCAGAATTCAAGGCTCCCACCTTC-3′. This fragment was cloned into the pMC86 vector (Chevray and Nathans, 1992) and confirmed by sequencing. The construction of the pMC86 bait vector and yeast transformation and selection were previously described (Chern et al., 2001).

To generate the XA21K668 construct for the yeast two-hybrid analyses, XA21K668 was first PCR-amplified using the primer pair 5′-CACCATGTCATCACTCTACTTGCTTA-3′/5′-TCAGAATTCAAGGCTCCCA-3′ and cloned into the pENTR™/D-TOPO vector (Invitrogen). After confirmation by DNA sequencing, the positive clone was cloned into the yeast two-hybrid vector pNlexA carrying a Gal4 BD domain (Clontech, Mountain View, CA, USA) through LR recombination (Invitrogen). To generate the XA21K736E construct, a single amino acid mutation was introduced into XA21K668 by site-directed mutagenesis using primers 5′-GTTGCAGTGGAAGTACTAAAGCTTGAAAATCC-3′ and 5′- CTTTAGTACTTCCACTGCAACATGATCTTGGATA-3′. To generate the XA21K(TDG) construct, a truncated form of XA21 was amplified using primers 5′-CACCATGACAGATGGTTTCGCGCCGACC-3′/-TCAGAATTCAAGGCTCCCA-3′. The amplified fragments were cloned into the pNlexA vector carrying a Gal4 BD domain (Clontech), respectively, following the same procedure as XA21K668.

To make the OsWRKY62 construct for the yeast two-hybrid analysis, *OsWRKY62.1* cDNA was sub-cloned from its pENTR™/D-TOPO construct version into the pB42ADB2 vector using the Gateway® LR clonase (Invitrogen). The pB42ADB2 vector was generated by adding an additional reading frame cassette B into pB42AD vector (Clontech) according to the Gateway® Vector Conversion System instruction manual (Invitrogen).

Phylogenetic Analysis

Plant WRKY IIa subfamily members with high similarity to OsWRKY62 were identified by BLAST search. To generate phylogenetic tree, the conserved WRKY domains were aligned with the ClustalW program and then used for neighbor-joining phylogenetic tree construction by using the MEGA 4 program (Tamura et al., 2007). Ten thousand bootstrap replicates were performed.

Generation of Fusion Proteins and Subcellular Localization

The full-length OsWRKY62.1 and OsWRKY62.2 cDNAs excluding the stop codon were amplified using primer sets 5′-GGGATCCCATGGACGACGACGGCGACGGC-3′/5′- GGGATCCCAAATGAACAGGAATGTGTGG-3′ and 5′-GGGATCCGATGGAGGAGAACGCGCGGCTG-3′/5′- GGGATCCCAAATGAACAGGAATGTGTGG-3′, respectively, and cloned into the pGEM T Easy vector (Promega, Madison, WI, USA). The inserts were then cut with BamHI and sub-cloned in-frame to the coding region of soluble modified green fluorescent protein (smGFP2) under the control of the maize ubiquitin promoter (Park et al., 2006). The constructs were introduced into rice protoplasts using a PEG–mediated transformation method (Bart et al., 2006). The protoplasts were viewed at 24–50 h after transformation with a fluorescent microscope (Leica, Bannockburn, IL, USA) using a 40-fold objective and either bright field, a GFP filter (filter set 38 HE with excitation: BP470/40; beamsplitter: FT 495; and emission: BP 525/50) or a RFP filter (filter set 31 with excitation: BP565/30; beamsplitter: FT 585; and emission: BP 620/60). Images were obtained using Nikon capture control.

PCR Analyses

The presence of the OsWRKY62.1ox transgene in genomic DNA was examined by PCR using primers 5′-AGGATCTTGAGGAGAAGCTGA-3′/5′-TGTGTGGGATTTGATCCACAA-3′, which amplify an 850-bp cDNA fragment and a 1.47-kb genomic fragment.

RNA Extraction and RT–PCR Analyses

Total RNA was extracted from leaves using Trizol reagent (Invitrogen). Total RNA was converted into cDNA using M-MLV reverse transcriptase (EC 2.7.7.49; Invitrogen). PCR analyses were performed with gene-specific primer sets: 5′- ATGGACGACGACGGCGACGGCT-3′/5′-GCGGTCGGCGGCTGCTGTCTC-3′ (for *OsWRKY62.1*); 5′-GCAGGGAGCGTATACAAGACCAA-3′/5′-CACGCCACAGTAACATGACCACAA-3′ (for *Betv1*); 5′-TTATCCTGCTGCTTGCTGGT-3′/5′-GGTCGTACCACTGCTTCTCC-3′ (for *PR1a*), 5′-CGCAGCTCACATTATCAAGTCAGA-3′/5′-GAAGCAGCAATACGGAGATGGATG-3′ (for *PR10*); 5′-ATGGCTCCGGCCTGCGTCTCCGA-3′/5′-GGCATATTCGGCAGGGTGAGCGA-3′ (for *PBZ1*). Primers 5′-GCTCGAGCTTAGCTGCCGCCATGGAC-3′/5′-CACCTTCTGCCCGTACTTCCTCCATTG-3′ were used to detect *OsWRKY62.1* (488-bp) and *OsWRKY62.2* (606-bp) simultaneously in one reaction. Primers 5′-ATCACACTCGACCTGACGAA-3′/5′-CAGGAATGTGTGGGATTTGA-3′ were used to amplify a 223-bp region present in both *OsWRKY62.1* and *OsWRKY62.2*. 18S ribosomal RNA was used as an internal control following the QuantumRNA 18S Internal Standards

manual (Ambion, Austin, TX, USA). The amplified products were then resolved by gel electrophoresis.

Accession Numbers

Sequence data from this article can be found in the GenBank data libraries under accession numbers as follows: OsWRKY62.1, NP_001063185; OsWRKY62.2, DAA05127; OsWRKY76, DAA05141; OsWRKY71, DAA05136; OsWRKY28, DAA05093; NtWIZZ, BAA87058; PcWRKY4, AF204925; CaWRKY1, AAX20040; VaWRKY4, AAR37421; AtWRKY18, NP_567882; AtWRKY 40, NP_178199; AtWRKY60, NP_180072; AtWRKY22, NF_192034; AtWRKY29, NP_194086; GmWRKY27, ABC26917; HvWRKY1, CAD60651; HvWRKY2, CAH68818; GhWRKY2, ABI23959; BrWRKY1, AAX76840; AfABF2, CAA88331; PR1a, ABK55608; PR10, BAD03969; PBZ1, BAA24277; Betv1, NP_001066995.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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