Differential Requirement of *Oryza sativa* RAR1 in Immune Receptor-Mediated Resistance of Rice to *Magnaporthe oryzae*

Min-Young Song^{1,5}, Chi-Yeol Kim^{1,5}, Muho Han¹, Hak-Seung Ryu¹, Sang-Kyu Lee¹, Li Sun², Zuhua He², Young-Su Seo^{3,4}, Patrick Canal⁴, Pamela C. Ronald^{1,4}, and Jong-Seong Jeon^{1,*}

The required for *Mla12* resistance (RAR1) protein is essential for the plant immune response. In rice, a model monocot species, the function of *Oryza sativa* RAR1 (*OsRAR1*) has been little explored. In our current study, we characterized the response of a rice *osrar1* T-DNA insertion mutant to infection by *Magnaporthe oryzae*, the causal agent of rice blast disease. *osrar1* mutants displayed reduced resistance compared with wild type rice when inoculated with the normally virulent *M. oryzae* isolate PO6-6, indicating that *OsRAR1* is required for an immune response to this pathogen. We also investigated the function of *OsRAR1* in the resistance mechanism mediated by the immune receptor genes *Pib* and *Pi5* that encode nucleotide binding-leucine rich repeat (NB-LRR) proteins. We inoculated progeny from *Pib/osrar1* and *Pi5/osrar1* heterozygous plants with the avirulent *M. oryzae* isolates, race 007 and PO6-6, respectively. We found that only *Pib*-mediated resistance was compromised by the *osrar1* mutation and that the introduction of the *OsRAR1* cDNA into *Pib/osrar1* rescued *Pib*-mediated resistance. These results indicate that *OsRAR1* is required for *Pib*-mediated resistance but not *Pi5*-mediated resistance to *M. oryzae*.

INTRODUCTION

The plant innate immune response is mediated by cell surface and cytoplasmic receptors (Boller and He, 2009; Lee et al., 2006; 2009a; Thomma et al., 2011). In plants, the cell surface recognition of conserved microbial signatures, also known as pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs), is mediated by pattern recognition receptors (PRRs) that often carry kinases of the non-arginine aspartate (RD) class (Bittel and Robatzek, 2007; Dardick and Ronald, 2006; Lee et al., 2006; Zipfel et al., 2004). Plants also contain a set of intracellular receptors, the nucleo-

tide binding-leucine rich repeat (NB-LRR) proteins. Plant NB-LRR proteins typically directly or indirectly recognize pathogen-derived proteins, also called effectors that trigger resistance to specific strains of the microbe. This resistance is often associated with a hypersensitive response. Both PRRs and NB-LRRs have been widely used in the agricultural breeding of important crop plants to generate resistance (Boller and He 2009; Jones and Dangl, 2006; Martin et al., 2003).

The plant immune response requires several downstream components including RAR1 (required for *Mla12*-mediated resistance). In *Arabidopsis*, *rar1* mutations lead to the enhanced growth of the virulent strain *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (Holt et al., 2005). In barley, the loss of *RAR1* promotes susceptibility in the *mlo-5* genetic background to the *Magnaporthe oryzae* fungus (Jarosch et al., 2005). These data collectively indicate that *RAR1* is required for plant pathogen resistance in diverse genetic backgrounds and species. Consistently, the overexpression of the rice ortholog of RAR1, known as *Oryza sativa* RAR1 (*OsRAR1*), significantly increases the resistance of this plant to *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal agent of bacterial leaf blight, and to *M. oryzae* (Wang et al., 2008).

RAR1, encoding a eukaryotic zinc-binding protein, was originally identified in barley due to its requirement for the *Mla12*-mediated resistance to powdery mildew (Shirasu et al., 1999). It was later found to be essential for *Mla6*, but not *Mla1*, resistance (Azevedo et al., 2002). In *Arabidopsis*, *RAR1* is required for the resistance to *P. syringae* mediated by the *Rpm1*, *Rps2*, and *Rps4* genes, and is also required for the resistance to *Hyaloperonospora arabidopsidis* mediated by *Rpp4*. In contrast, neither the *Rpp2*- nor *Rpp8*-mediated resistance to *H. arabidopsidis* requires *RAR1* (Austin et al., 2002; Holt et al., 2005; Muskett et al., 2002; Tomero et al., 2002). These findings indicate that *RAR1* contributes differentially to NB-LRR-mediated resistance responses. Recent studies have demonstrated that RAR1 forms a complex with the molecular chaperones HSP90

¹Graduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea, ²National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China, ³Department of Microbiology, Pusan National University, Busan 609-735, Korea, ⁴Department of Plant Pathology and the Genome Center, University of California, Davis, CA 95616, USA, ⁵These authors contributed equally to this work.

*Correspondence: jjeon@khu.ac.kr

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Table 1. PCR primers used in this study

Name	Forward (5' → 3')	Reverse (5' → 3')	Amplicon size (bp)*	Annealing temp (°C)
Nbs	ATCAACTCTGCCACAAATCC	CCCATATCACCACCTTGTCCCC	538 (gDNA)	58
Pib	GCTTGATTGTTCTAGATGATTTCT	ATCTGATCAAGGTGATGATATTCT	979 (cDNA)	56
OsRAR1 (cDNA)	TCCATATGTCGACGGAGGCGGAGACC	CGGGATCCAAGATGGGGCATGGTGAA	702 (cDNA)	56
OsRAR1-RT	AAAGCAGTTCCTACTAAACCATTA	CTGAGCTCTCATGATTATCCTTCTCCTTGTAG	278 (cDNA) 454 (gDNA)	58
OsRAR1	AGAGGGTACTTCTAGAAGGATGCA	ATGGGTGTTACCAAAAGTCTGCAG	1,354 (gDNA)	65
JJ80-T3	TTATGAGATTAGGAGTGTAT	ATGTAAAGGCAAAAGCTGAT	442 (gDNA)	56
JJ113-T3	CTCTTGATGATCTTTGTTAC	GGATGATGTGATCTGCAGAG	484 (gDNA)	56
Pi5-1	TACAAGTTGGCAGCTTTATCTGAG	TCAGAAGCACTGGATCTTTCTGCA	524 (722)	56
Pi5-2	AGTGAACCTCAAACATGTGAACAC	TCATACCTGTTGCGGTTTCTGCCT	798 (2085)	56
2715RB		ATCCAGACTGAATGCCACAGG	N/A**	65
Ubq5	GACTACAACATCCAGAAGGAGTC	TCATCTAATAACCAGTTCGATTTC	795 (cDNA)	58
<i>M. oryzae</i> 28S rDNA	TACGAGAGGAACGCGTCATTCAGATAATTA	TCAGCAGATCGTAACGATAAAGCTACTC	330 (gDNA)	58
Ubq1	GTGGTGGCCAGTAAGTCCTC	GGACACAATGATTAGGGATCA	246 (cDNA)	56

*PCR products amplified from cDNA and genomic DNA (gDNA) as templates.

**Not applicable

(heat shock protein 90) and SGT1 (suppressor of G2 allele of *skp1*) (Kadota et al., 2010; Shirasu, 2009).

Rice blast is one of the most devastating diseases for this vital global crop (Ou, 1985). To date, a total of 14 rice blast resistance genes have been cloned and characterized (Liu et al., 2010; Okuyama et al., 2011). With the exception of *Pi-d2*, a non-RD receptor-like kinase (Chen et al., 2006), these immune receptors all encode NB-LRR type proteins. Despite the importance of these proteins in the immune response, the molecular mechanisms underlying the activation of NB-LRR-mediated resistance remain largely unknown. In particular, the role of the molecular chaperone complex necessary for NB-LRR-mediated resistance to *M. oryzae* in rice has not yet been explored.

To examine the effects of the *OsRAR1* mutation on rice NB-LRR gene-mediated resistance to *M. oryzae*, we isolated a T-DNA insertion mutant line, *osrar1*, and generated two monogenic resistance lines IRBLb-B carrying *Pib* and IRBL5-M carrying *Pi5* in the *osrar1* background. We inoculated *Pib/osrar1* and *Pi5/osrar1* plants with their respective avirulent *M. oryzae* isolates, and found that the *osrar1* mutation compromises *Pib*-mediated resistance but not *Pi5*-mediated resistance.

MATERIALS AND METHODS

Plant materials and growth conditions

The rice (*Oryza sativa* L.) cultivars (cvs.) Dongjin and Lijiangxintuanheigu (LTH) and two monogenic resistance lines, IRBLb-B carrying *Pib* and IRBL5-M carrying *Pi5* (Tsunematsu et al., 2000; Yi et al., 2004), were obtained from the National Crop Experiment Station, RDA, Suwon, Korea. Dongjin and LTH were used as the respective susceptible controls to the *M. oryzae* isolates, PO6-6 and race 007. All rice plants were grown in a greenhouse at 30°C during the day and at 20°C at night in a light/dark cycle of 14 h/10 h for inoculation and seed harvesting.

osrar1 mutant isolation and genetic crosses

A T-DNA-tagged *OsRAR1* mutant (cv. Dongjin) *osrar1* was identified by searching the rice T-DNA Insertion Sequence Database (An et al., 2005a; 2005b; Jeong et al., 2006; <http://signal.salk.edu/cgi-bin/RiceGE>). A homozygous mutant was isolated by genomic DNA PCR analysis. *OsRAR1*- (OsRAR1-Forward (F)/Reverse (R)) and T-DNA-specific (2715RB-R) primers were used for the genotyping of *osrar1* (Table 1). PCR amplification was performed in a final volume of 40 µl (100 pmol of each primer, 20 µM each of dNTPs, 10 mM Tris-HCl pH 9.0, 2 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, and 0.5 U of Taq polymerase) using 50 ng of genomic DNA as template. The amplification conditions were as follows: 94°C for 5 min followed by 35 cycles of 94°C, 1 min; 56°C, 1 min; and 72°C, 1 min, with a final extension at 72°C for 5 min.

To generate monogenic resistance lines in the background of *osrar1*, we crossed the homozygous *osrar1* mutant with the monogenic rice lines IRBLb-B and IRBL5-M, respectively, and further grew the F₁ plants to obtain self-pollinated F₂ seeds.

DNA extraction and genotypic analysis

Total genomic DNAs were extracted from the young leaves of F₂ seedlings following the protocol described by Chen and Ronald (1999). To determine the genotypes of individual F₂ plants, we designed the *Pib*-specific primers (Nbs-F/R) using the *Pib* genomic DNA sequence (GenBank accession no. AB013448; Table 1). The sequence characterized amplified region (SCAR) markers JJ80-T3-F/R and JJ113-T3-F/R (Table 1; Yi et al., 2004) were used to determine the presence of *Pi5* in genomic DNA. Genotyping of *OsRAR1* and PCR amplifications were performed as described previously.

Pathogen inoculation and disease evaluation

The *M. oryzae* isolates 007 and PO6-6 were used to evaluate *Pib*- and *Pi5*-mediated resistance, respectively (Lee et al., 2009b;

Fig. 1. Isolation and characterization of the *osrar1* mutant. (A) Schematic diagram of the rice *OsRAR1* gene and the position of the T-DNA insertion. In *osrar1*, the T-DNA is inserted into the fourth exon. The arrows indicate primers for genotypic analysis and RT-PCR. The five exons are indicated by boxes. (B) Genomic DNA PCR analysis of the *osrar1* mutant. PCR products amplified from *OsRAR1*-F/R primers indicate the presence of the wild type (W) copy of *OsRAR1*. PCR products amplified using *OsRAR1*-F and T-DNA 2715RB primers indicate the presence of the T-DNA inserted mutation (t). (C) RT-PCR analysis of the homozygous *osrar1* mutant (t/t). *OsRAR1* transcripts are not detectable in the homozygous mutant. *Ubq5* was amplified as a PCR internal control.

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Noda et al., 1999; Wang et al., 1999). All inoculations and disease evaluations were conducted in the greenhouse facilities at Kyung Hee University using the following two inoculation methods. First, *M. oryzae* 007 and PO6-6 were grown on oatmeal agar medium for two weeks at 24°C in the dark and their conidia were induced four days before collection by scratching the plate surface with a sterilized loop. Collected conidia were suspended in water containing 0.005% Tween 20 and adjusted to a concentration of 1×10^5 conidia/ml and then sprayed onto three week-old seedlings. Disease evaluation was carried out seven days after inoculation (Chen et al., 1996). Second, to quantify the resistance response of the *osrar1* mutant to *M. oryzae* PO6-6, agar blocks covered with spores were placed on injured spots of 2.0 mm diameter in second fully expanded leaves from the top of five week-old plants (Takahashi et al., 1999). All inoculated plants were placed in sealed containers to maintain humidity at 24°C in darkness for 24 h and then transferred to a growth chamber at 24°C and 80% humidity under a 14 h/10 h (light/dark) photoperiod. Blast lesion lengths were then measured 10 days after inoculation. *M. oryzae* DNA contents were determined by quantitative real-time PCR (qRT-PCR) of *M. oryzae* 28S rDNA relative to rice *Ubiquitin1* (*Ubq1*) DNA in rice leaves using specific primers (Table 1) (Eom et al., 2011; Qi and Yang, 2002).

RNA preparation and RT-PCR analysis

To examine the expression of *OsRAR1*, *Pib*, and *Pi5* in leaves from the *osrar1* mutant and progeny plants of the crossed lines, *Pib/osrar1* and *Pi5/osrar1*, total RNAs were prepared using Trizol reagent and reverse-transcribed with an oligo(dT) primer and a First Strand cDNA Synthesis kit (Roche) (Cho et al., 2006). First-strand cDNAs were used in PCR reactions with gene-specific primers for *OsRAR1*, *OsRAR1* RT-F/R; *Pib*, *Pib*-F/R; and *Pi5*, *Pi5*-1-F/R and *Pi5*-2-F/R (Table 1). Primers (*Ubq5*-F and *Ubq5*-R) for the rice *Ubiquitin5* (*Ubq5*) gene (Jain et al., 2006) were used as internal PCR control (Table 1). PCR amplifications were performed as described previously.

Genetic complementation experiment

A cDNA fragment encoding full-length *OsRAR1* was amplified from a rice cDNA library using the primers, 5'-TCCATATGT-CGACGGAGGCGGAGACC-3' and 5'-CGGGATCCAAGATG-GGGCATGGTGAA-3' (Table 1). The PCR product was cloned into the pGEM-T easy vector (Invitrogen) and the insert was confirmed by sequencing. For expression in rice, the *OsRAR1* cDNA was subcloned from the pGEM-T easy vector using the

SpeI and *Bam*HI sites into the Ubi/NC4300 binary vector carrying the *phosphomannose isomerase* (*PMI*) gene as a selectable marker (Eom et al., 2011). Rice transformations were conducted as described previously using the homozygous *Pib/Pib/osrar1/osrar1* line (Eom et al., 2011). The resulting binary vector was introduced into *Agrobacterium tumefaciens* EHA105 and used to infect rice calli for transformation. The expression of *OsRAR1* in transgenic plants was confirmed by RT-PCR as described previously.

Western blotting

To prepare total protein extracts, leaves of one month-old rice plants were ground into powder in liquid nitrogen, thawed in cold extraction buffer (150 mM Tris-HCl, pH 7.5, 6 M urea, 2% sodium dodecyl sulfate, and 5% β -mercaptoethanol), and put on ice for 10 min. The extracts were then put in boiling water for 5 min and centrifuged at $14,000 \times g$ at 4°C for 10 min. The supernatants were next mixed with equal volumes of 2 $\times$ SDS loading buffer, boiled in water for 5 min and stored at -20°C. The concentration of extracted proteins was determined using the Bradford Protein Assay Kit (Pierce, USA). For western blotting, the proteins were separated using 10% SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. The RAR1 antibody was used according to Wang et al. (2008) at a dilution of 1:1,000. The secondary antibody was goat anti-rabbit IgG (H+L) (CWBIO: www.cwbio.com) and was diluted 1:5000. Signals were developed using the ECL western blot kit (CWBIO).

RESULTS

Evaluation of the immune response to *M. oryzae* in the *osrar1* rice mutant

Recent evidence suggests that resistance to virulent pathogens in *Arabidopsis* and barley is dependent on the level of the RAR1 protein (Holt et al., 2005; Jarosch et al., 2005). We previously showed that *OsRAR1* functionally rescues the disease resistance of the *atar1* mutant to the virulent *P. syringae* pv. *tomato* DC3000. In addition, overexpression of *OsRAR1* in rice has been shown to result in enhanced resistance to all the four tested virulent *M. oryzae* isolates as compared with wild type (Wang et al., 2008).

To confirm the role of *OsRAR1* in the immune response in the rice plant, we isolated an *osrar1* knockout mutant from our T-DNA mutant population (An et al., 2005a; 2005b; Jeong et al., 2006). The isolated mutant allele, *osrar1*, harbors a T-DNA

Fig. 2. Resistance responses of the *osrar1* mutant to a virulent *M. oryzae* isolate PO6-6. (A) Disease response 10 days after the punch-inoculation of wild type (WT) and the T-DNA mutant *osrar1*. (B) Quantitative leaf lesion length of wild type and *osrar1* 10 days after the inoculation. *osrar1* becomes more susceptible compared to WT. (C) Ratios of *M. oryzae* 28S rDNA versus rice *Ubq1* DNA determined by qRT-PCR. Higher disease severity was observed in *osrar1*. Each datapoint represents the mean values with standard deviations ($\pm$ SD) from at least fifteen different plants. * $P < 0.01$ (Student's *t*-test).

Fig. 3. Resistance responses of *Pib/osrar1* and *Pi5/osrar1* F₂ plants to avirulent *M. oryzae* isolates. (A, Top) F₂ individual plants derived from *Pib/osrar1* were inoculated with *M. oryzae* isolate 007 using a spray method. Notably, *Pib* $\pm$ /*osrar1*/*osrar1* plants are susceptible to the *M. oryzae* isolate 007. In contrast, *Pib* $\pm$ /*OsRAR1* $\pm$ plants are resistant. *Pib*-lacking plants were also found to be susceptible. LTH and IRBLb-B are susceptible and resistant controls, respectively. (A, Bottom) RT-PCR analysis of *Pib* and *OsRAR1* from the leaves of three-week old plants at seven days after spray inoculation. *Ubq5* was amplified as a PCR internal control. (B, Top) F₂ individual plants derived from *Pi5/osrar1* were inoculated with *M. oryzae* isolate PO6-6 using the spray method. *Pi5* $\pm$ /*osrar1*/*osrar1* and *Pi5* $\pm$ /*OsRAR1* $\pm$ are all resistant to this pathogen. In contrast, *Pi5*-lacking plants were found to be susceptible. LTH and IRBL5-M were used as susceptible and resistant controls, respectively. (B, Bottom) RT-PCR analysis of *Pi5-1*, *Pi5-2*, and *OsRAR1* from the leaves of three-week old plants at seven days after spray inoculation. *Ubq5* was amplified as a PCR internal control. All experiments were repeated three times with similar results.

insertion in the fourth exon of *OsRAR1* (Fig. 1A). Using *OsRAR1*-specific and T-DNA-specific primers, we isolated mutants that were homozygous for the insertion from the segregating progeny (Fig. 1B). RT-PCR analysis indicated that endogenous *OsRAR1* transcript is absent in *osrar1*, confirming it is a null mutant (Fig. 1C). We then inoculated the *osrar1* mutant with a virulent *M. oryzae* isolate PO6-6 to determine whether the loss of function of *OsRAR1* affects disease resistance. Using the spot inoculation method, we consistently found that the *osrar1* null mutant displays enhanced susceptibility to PO6-6 (Fig. 2A). The average lesion size of *osrar1* mutant was found to be 3

mm longer than that of wild type 10 days after inoculation (Fig. 2B). In qRT-PCR analysis, ratios of *M. oryzae* 28S rDNA versus rice *Ubq1* DNA were significantly higher in *osrar1* than those in wild type at 10 days after inoculation (Fig. 2C). These results are consistent with our previous finding (Wang et al., 2008) that *OsRAR1* is a positive regulator of the rice immune response.

Function of *OsRAR1* in immune receptor-mediated resistance to *M. oryzae*

We previously showed that *OsRAR1* restores the *RPS2*-media-

Fig. 4. *OsRAR1* genetic complementation experiments. (A) The vector used for the *OsRAR1* complementation test. The Ubi/NC4300 binary vector carrying the CaMV35S promoter (P_{35S}):the phosphomannose isomerase (PMI) gene fusion as a selectable marker was used. The maize ubiquitin1 promoter (P_{ZmUbi1}) was used to express the *OsRAR1* cDNA. Tnos, *nopaline synthase* terminator. (B) Disease phenotype after seven days elapsed from the spray inoculation of complemented transgenic plants (OsRAR1-ox). The genetic background *Pib/Pib/osrar1/osrar1* was used as a control (Con). (C) Genotype analysis of the control and OsRAR1-ox lines. Nbs-F/R PCR products indicate the presence of the *Pib* gene. OsRAR1 RT-F/R PCR amplifies 454-bp genomic DNA and 278-bp cDNA products. PCR products from OsRAR1-F/R primers indicate the presence of a wild type copy of *OsRAR1*. PCR products from OsRAR1-F and T-DNA 2715RB primers indicate the presence of the T-DNA inserted mutation. (D) RT-PCR analysis of the control and OsRAR1-ox lines. Whereas the *OsRAR1* transcript was not detectable in the control, the complemented line expressed this gene. *Pib* was found to be expressed in all lines. *Ubg5* was amplified as a PCR internal control.

ted resistance of *atar1* to the avirulent pathogen *P. syringae* pv. *tomato* DC3000 (*AvrRpt2*) to the same level as wild type *Ler* (Wang et al., 2008). To examine whether *OsRAR1* functions in NB-LRR gene-mediated resistance in rice, we utilized two genes, *Pib* (Wang et al., 1999) and *Pi5* (Lee et al., 2009b), which confer race-specific resistance to *M. oryzae* and encode NB-LRR proteins. IRBLb-B carrying *Pib* and IRBL5-M carrying *Pi5* (Tsunematsu et al., 2000; Yi et al., 2004) were crossed with *osrar1*. The resulting F₁ hybrid rice lines each contain a single NB-LRR gene and the mutant *osrar1* allele. Subsequently, F₂ progeny plants were obtained from the self-pollinated F₁ plants.

To evaluate disease resistance, we inoculated the segregating F₂ individual plants with *M. oryzae* isolates 007 and PO6-6, avirulent to IRBLb-B and IRBL5-M, respectively, in a spray inoculation experiment (Figs. 3A and 3B, top). All *Pib/±/osrar1/osrar1* F₂ individuals were found to be susceptible to *M. oryzae* isolate 007, to a similar level to that observed for the susceptible Lijiangxintuanheigu (LTH) control. In contrast, all *Pib/±/OsRAR1/±* F₂ individuals were as resistant as the control IRBLb-B line. In addition, all of the F₂ individuals lacking the *Pib* gene were observed to be highly susceptible to *M. oryzae* 007 (Fig. 3A, top).

We also inoculated the segregating F₂ individual plants derived from the *Pi5/osrar1* cross with *M. oryzae* isolate PO6-6 using the same spray inoculation method (Fig. 3B, top). All *Pi5/±/osrar1/osrar1* F₂ individuals maintained resistance. In addition, all *Pi5/±/OsRAR1/±* F₂ individuals showed resistance at a similar level to the control IRBL5-M line. In contrast, all of individuals lacking the *Pi5* gene were highly susceptible to *M. oryzae* PO6-6 (Fig. 3B, top). We next carried out RT-PCR experiments to assess the expression levels of *Pib*, *Pi5* and *OsRAR1*. We extracted total RNAs from the leaves of three-week old plants seven days after inoculation. Each gene was found to

be expressed in all of the genetic backgrounds (Figs. 3A and 3B, bottom). These results indicate that *OsRAR1* is necessary for *Pib*-mediated resistance but not for *Pi5*-mediated resistance, and highlights a differential requirement of *OsRAR1* in rice immune receptor-mediated resistance.

Genetic complementation assay of *OsRAR1*

To further assess whether the *osrar1* mutation is directly responsible for the loss of *Pib*-mediated resistance in rice, we carried out an *OsRAR1* complementation experiment. We introduced *OsRAR1* cDNA, isolated from a Dongjin rice cultivar cDNA library, under the control of the maize ubiquitin1 promoter (P_{ZmUbi1}), into *Pib/Pib/osrar1/osrar1* via *Agrobacterium*-mediated transformation (Eom et al., 2011; Fig. 4A).

Transgenic rice plants were selected on mannose-containing media and grown for seed. T₁ progeny from the transgenic plants were subsequently inoculated with *M. oryzae* isolate 007. The *Pib/Pib/osrar1/osrar1* line expressing *OsRAR1* cDNA regained resistance to *M. oryzae* isolate 007. In contrast, the null segregant plants carrying the *Pib/Pib/osrar1/osrar1* genotype showed susceptibility to this pathogen (Fig. 4B). Genomic DNA PCR and RT-PCR experiments confirmed that the *Pib/Pib/osrar1/osrar1* transgenic plants expressed both the *OsRAR1* cDNA and *Pib* (Figs. 4C and 4D). Western blotting of the transgenic plants with anti-*OsRAR1* revealed that the *Pib/Pib/osrar1/osrar1* line expressing the *OsRAR1* cDNA also expressed a high level of *OsRAR1* protein and the null segregant plants lacking the *OsRAR1* cDNA showed no *OsRAR1* protein (Fig. 5). These results demonstrate that the *OsRAR1* gene is essential for the *Pib*-mediated resistance of rice to *M. oryzae*.

Fig. 5. Western blotting analysis of complemented OsRAR1-ox transgenic lines. (A) The blot was probed with an RAR1 primary antibody. OsRAR1-ox lines produce the OsRAR1 proteins. In contrast, the control (Con) lacks this protein. (B) Coomassie-stained gel indicating equal loading.

DISCUSSION

OsRAR1 is a positive regulator of the rice immune response

RAR1 is required for the resistance of *Arabidopsis* to the virulent pathogen *P. syringae* pv. *tomato* (Pst) DC3000 (Holt et al., 2005) and of barley to the virulent *M. oryzae* isolate (Jarosch et al., 2005). In rice, the overexpression of *OsRAR1* increases the resistance to the virulent *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *M. oryzae* strains (Wang et al., 2008). In our present study, we examined the role of *OsRAR1* using null mutation lines. Our quantitative inoculation experiments clearly demonstrated that the loss of *OsRAR1* enhances the susceptibility to the virulent *M. oryzae* PO6-6 fungal pathogen (Fig. 2). We also found that *osrar1* mutants had significantly increased susceptibility to Xoo PXO99 (Seo et al., 2011). Hence, our studies in rice further indicate a positive role for *RAR1* in the plant immune response.

OsRAR1 is required for *Pib*- but not *Pi5*-mediated resistance to *M. oryzae*

RAR1 forms complexes with the molecular chaperones HSP90 and SGT1 to initiate a signaling cascade in diverse plant immune responses (Kadota et al., 2010; Seo et al., 2008; Shirasu, 2009; Shirasu and Schulze-Lefert, 2003). The function of these proteins in disease resistance responses have been extensively investigated previously using mutant analyses (Austin et al., 2002; Chandra-Shekhara et al., 2004; Hubert et al., 2003; Lu et al., 2003; Shirasu et al., 1999; Takahashi et al., 2003) and by virus induced gene silencing (VIGS) analyses in several plant species (Bhattarai et al., 2007; de la Fuente van Bentem et al., 2005; Leister et al., 2005; Liu et al., 2004; Scofield et al., 2005). The data from these studies revealed differing specificities for

these proteins in diverse NB-LRR-mediated resistance responses. For example, *RAR1* and *SGT1* are required for *MLA6*- and *MLA12*-, but not *MLA1*-, mediated resistance (Azevedo et al., 2002). Tobacco N, a *Tobacco mosaic virus* resistance protein, and wheat Lr21, a *Puccinia triticina* resistance protein, require each of the proteins for the initiation of immune responses against pathogens (Liu et al., 2004; Lu et al., 2003; Peart et al., 2002; Scofield et al., 2005). The function of each of these chaperone proteins has not yet been fully explored in rice immune responses governed by NB-LRRs.

In the present study, we addressed the function of *OsRAR1* in rice through an analysis of two NB-LRR genes, *Pib* (Wang et al., 1999) and *Pi5* (Lee et al., 2009b). *Pi5*-mediated resistance requires two adjacent typical NB-LRR genes *Pi5-1* and *Pi5-2* (Lee et al., 2009b). Our current results demonstrate that *OsRAR1* is necessary for the *Pib*-mediated resistance to *M. oryzae* but not for *Pi5*-mediated resistance to this pathogen. These results are in agreement with previous results demonstrating that *RAR1* differentially contributes to NB-LRR gene function (Kadota et al., 2010; Seo et al., 2008; Shirasu, 2009; Shirasu and Schulze-Lefert, 2003).

The fact that only a subset of NB-LRR proteins is affected by *rar1* mutations has been explained by a “threshold model”. That is, when destabilized in *rar1* mutant background, *RAR1*-independent proteins like *Pi5* might accumulate at relatively high steady-state levels that are above the threshold required for efficient defense responses (Bieri et al., 2004). In contrast, *RAR1*-dependent proteins like *Pib* might be present at relatively lower levels than this critical threshold in *rar1* mutants. In this regard, it is noteworthy that *Pi5-1* and *Pi5-2* are constitutively and relatively highly expressed before and after pathogen challenges (Lee et al., 2009b), whereas the *Pib* transcript is only weakly detectable upon the pathogen inoculation (Wang et al., 1999). To address this hypothesis, further analyses are needed to determine levels of *Pi5* and *Pib* proteins in the presence or absence of *OsRAR1*.

The differential dependence of immune responses on the *RAR1* gene has been observed also for *SGT1* and *HSP90*. For instance, it has been shown in *Arabidopsis* that *RAR1* and *HSP90*, but not *SGT1*, are required for *RPM1*-, *RPS2*-, and *RPS4*-mediated immune responses (Austin et al., 2002; Hubert et al., 2003; Takahashi et al., 2003). Similarly, *RPP2*-mediated resistance requires *SGT1* but not *RAR1*. In the *RPP4*-mediated resistance pathway, *SGT1* and *RAR1* are employed together, whereas *RPP8* does not require either of these proteins for a disease resistance response (Austin et al., 2002). Based on this differential contribution of molecular chaperone components to resistance in other plant species, and our current observation of a differential requirement for *RAR1*, we hypothesize that *SGT1* and *HSP90* might also contribute differentially to rice immune receptor-mediated resistance.

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