Rice OsPAD4 functions differently from Arabidopsis AtPAD4 in host-pathogen interactions

Yinggen Ke, Hongbo Liu, Xianghua Li, Jinghua Xiao and Shiping Wang*
National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China

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*For correspondence (e-mail swang@mail.hzau.edu.cn).

SUMMARY
The extensively studied Arabidopsis phytoalexin deficient 4 (AtPAD4) gene plays an important role in Arabidopsis disease resistance; however, the function of its sequence ortholog in rice is unknown. Here, we show that rice OsPAD4 appears not to be the functional ortholog of AtPAD4 in host-pathogen interactions, and that OsPAD4 encodes a plasma membrane protein but that AtPAD4 encodes a cytoplasmic and nuclear protein. Suppression of OsPAD4 by RNA interference (RNAi) increased rice susceptibility to the biotrophic pathogen Xanthomonas oryzae pv. oryzae (Xoo), which causes bacteria blight disease in local tissue. OsPAD4-RNAi plants also show compromised wound-induced systemic resistance to Xoo. The increased susceptibility to Xoo was associated with reduced accumulation of jasmonic acid (JA) and phytoalexin momilactone A (MOA). Exogenous application of JA complemented the phenotype of OsPAD4-RNAi plants in response to Xoo. The following results suggest that OsPAD4 functions differently than AtPAD4 in response to pathogen infection. First, OsPAD4 plays an important role in wound-induced systemic resistance, whereas AtPAD4 mediates systemic acquired resistance. Second, OsPAD4-involved defense signaling against Xoo is JA-dependent, but AtPAD4-involved defense signaling against biotrophic pathogens is salicylic acid-dependent. Finally, OsPAD4 is required for the accumulation of terpenoid-type phytoalexin MOA in rice-bacterium interactions, but AtPAD4-mediated resistance is associated with the accumulation of indole-type phytoalexin camalexin.

Keywords: bacterial blight, bacterial streak, jasmonate, systemic resistance, Oryza sativa, Xanthomonas oryzae.

INTRODUCTION
Plants live in a complex environment. To defeat attempted pathogen invasion, plants use effective defense responses at the infection site and in systemic tissues. At the infection site, plants use the following two-tiered innate immune system to protect themselves: (i) pathogen-associated molecular pattern-triggered immunity (PTI), microbe-associated molecular pattern-triggered immunity or basal resistance; and (ii) effector-triggered immunity (ETI), gene-for-gene or race-specific resistance (Jones and Dangl, 2006; Thomma et al., 2011). PTI is initiated by plasma membrane-localized plant pattern recognition receptors; ETI is activated by cytoplasm-localized resistance (R) proteins (Jones and Dangl, 2006). As emerging data suggest that both plant pattern recognition receptors and R proteins can mediate a race-specific high level of resistance, or qualitative resistance, we named the gene conferring qualitative resistance the ‘major resistance’ (MR) gene (Thomma et al., 2011; Zhang and Wang, 2013). Plants use induced immunity to protect themselves from subsequent pathogen invasion in distal or systemic tissues after the first local infection; this type of immunity is separated into two classes: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Grant and Lamb, 2006). The phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene have been implicated as signals associated with systemic defense responses (Shah, 2009). The SAR pathway appears to partially overlap with PTI and ETI (Thomma et al., 2011). The accumulation of SA in distal tissue is the key for establishing SAR (An and Mou, 2011; Fu and Dong, 2013); however, JA as the systemic signal for SAR is controversial (Fu and Dong, 2013). Both JA-dependent and JA-independent SAR have been reported (Truman et al., 2007; Chaturvedi et al., 2008; Attaran et al., 2009). ISR frequently depends on the JA/ethylene signal pathway in both monocots and dicots (Shoresh et al., 2010; Balmer et al., 2013). It is controversial whether
SA can also function as the systemic signal for ISR. Barley ISR against biotrophic pathogens is SA dependent (Molitor et al., 2011), whereas Pseudomonas fluorescens WCS365r-induced Oryza sativa (rice) ISR against the hemibiotrophic Magnaporthe oryzae is dependent on the JA/ethylene-modulated signal, but is independent of SA signaling (De Vleesschauwer et al., 2008).

The SA-dependent and JA/ethylene-dependent pathways frequently interact either synergistically or antagonistically in plant-pathogen interactions (Durrant and Dong, 2004). Arabidopsis phytoalexin deficient 4 (AtPAD4), a pathogen and SA-induced defense-responsive gene, encodes a lipase-like protein and is required for the expression of multiple defense responses (Glazebrook et al., 1997; Zhou et al., 1998; Jirage et al., 1999). AtPAD4 functions in SA-dependent defense signaling by interacting with another lipase-like protein, enhanced disease susceptibility 1 (AtEDS1), in a positive feedback loop to promote SA biosynthesis, and it is required in some MR gene-mediated resistance to the biotrophic parasite Pseudomonas glycinea (Glazebrook et al., 1997; Falk et al., 1999; Feys et al., 2001; Wiemer et al., 2006). Induced expression of the pathogenesis-related 1 (PR1) gene is a marker of SAR in Arabidopsis (Uknes et al., 1992; Bowling et al., 1994). Pathogen-induced PR1 expression is AtPAD4 dependent, but exogenous SA-induced PR1 expression is AtPAD4 independent (Zhou et al., 1998). Disease resistance signaling involving AtPAD4, downstream of AtMPK4, may require the suppression of JA/ethylene signaling, however. The Arabidopsis pad4 mutant shows an increased susceptibility to the biotrophic pathogen Pseudomonas syringae pv. tomato DC3000, which is accompanied by induced expression of the marker gene PDF1.2 of JA/ethylene signaling (Jirage et al., 2001; Brodersen et al., 2006). Furthermore, the Arabidopsis HRT protein confers resistance to turnip crinkle virus, and SA-induced HRT expression is AtPAD4 dependent (Zhu et al., 2011); however, AtPAD4-mediated resistance to Myzus persicae (green peach aphid) is SA independent (Louis et al., 2012).

Bacterial blight caused by biotrophic Xanthomonas oryzae pv. oryzae (Xoo) is one of the most devastating diseases of rice worldwide (Kou and Wang, 2013). SA, JA and ethylene appear to have complex roles in the resistance of rice to Xoo. Previous studies have reported that the induction of defense-responsive genes involved in the resistance of rice to Xoo is accompanied by: an increased accumulation of SA and a reduced level of JA (Qiu et al., 2007; Xiao et al., 2009; Shen et al., 2010); an increased accumulation of JA, but not of SA (Tao et al., 2009); an increased accumulation of both SA and JA, but with a reduced level of ethylene (Tao et al., 2009; Shen et al., 2010, 2011); or reduced levels of SA and JA (Ding et al., 2008; Fu et al., 2011). The exact roles of SA and JA in the response of rice to Xoo infection remain to be elucidated, however.

Pathogen infection frequently influences the expression of rice OsPAD4, the sequence homolog of AtPAD4. OsPAD4 shows differential expression in resistant and susceptible plants during Xoo infection, suggesting the putative involvement of OsPAD4 in the response of rice to Xoo (Qiu et al., 2007; Ding et al., 2008; Tao et al., 2009; Xiao et al., 2009). To examine this inference, we generated OsPAD4-suppressing plants and analyzed the role of OsPAD4 in rice-Xoo interactions. These analyses confirmed that OsPAD4 regulates rice defense responses against Xoo and Xanthomonas oryzae pv. oryzicola (Xoc), which cause bacterial streak disease; however, OsPAD4 plays a different role in host-pathogen interactions compared with AtPAD4.

RESULTS

OsPAD4 encodes a plasma membrane protein

Sequence analysis showed that the sequence homolog of Arabidopsis AtPAD4 is the protein encoded by gene locus LOC_Os11g09010 in the rice genome. Thus, this gene is the sequence ortholog of AtPAD4, and we refer to it as OsPAD4 in this study. OsPAD4 (accession number: AK243523) is a single-copy gene in the rice genome. Its encoding protein and AtPAD4 share 26% sequence identity and 37% sequence similarity. The most similar regions between OsPAD4 and AtPAD4 are the predicted lipase regions, which cover approximately 215 amino acids, have 31% sequence identity and 38% sequence similarity, and harbor the catalytic triad of lipase, the conserved serine aspartate and histidine residues (Brady et al., 1990) (Figure S1).

Sequence analysis showed that PAD4 orthologs are conserved in at least 15 monocots and dicots (Figure S1); however, their predicted encoding proteins have different sizes ranging from 484 to 670 amino acids. According to amino acid sequence alignment, the partial lengths of the 15 PAD4 orthologs that continuously cover approximately 415 amino acids and harbor lipase regions and flanking sequences of the lipase regions were used to analyze the evolutionary relationship. The 15 PAD4 orthologs were classified into three major groups according to the phylogenetic tree (Figure 1a). OsPAD4 and its orthologs from other monocots were classified as group II, AtPAD4 was classified as group III and the PAD4 orthologs from other dicots were classified as group I. The results of bioinformatic analyses suggest that most of these PAD4 orthologs from different species are membrane proteins. The exceptions are AtPAD4 and wine grape PAD4, which are predicted to be soluble proteins and do not have putative transmembrane helixes. This prediction is support by the evidence that AtPAD4 localizes in the cytoplasm and nucleus (Feys et al., 2005).
To determine its subcellular localization, \( P_{35S}:OsPAD4-GFP \) was transiently expressed in rice protoplasts, with the fusion protein localized in the plasma membrane (Figure 1b). Transgenic rice plants carrying \( \mu_{35S}:OsPAD4-GFP \) were also generated. OsPAD4-GFP also localized in the plasma membrane of rice stem protoplasts isolated from these transgenic plants (Figure S2). These results confirm that OsPAD4 is a plasma membrane protein.

**Suppressing OsPAD4 increased rice susceptibility to Xoo**

To examine the role of OsPAD4 in rice-Xoo interactions, we suppressed its expression in susceptible rice variety Mudanjianqiang 8 by using an RNA interference (RNAi) strategy. Eight independent transgenic plants, D146RM1–D146RM8, were obtained. These T\(_0\) plants showed no obvious phenotypic changes during the developmental stage. All these T\(_0\) transgenic plants were inoculated with.eps
dn strain PXO61 at the booting (panicle development) stage. Seven of the eight plants showed an increased susceptibility to PXO61, with the disease area ranging from 61 to 100\% of the area infected in the wild-type variety (Figure 2a). The increased susceptibility was associated with suppressed expression of OsPAD4 in these transgenic plants; the correlation between disease area and OsPAD4 expression level in the T\(_0\) transgenic plants was \( 0.8833 \) (significant at \( \alpha = 0.01; n = 8 \)). Bacterial growth analysis showed that the growth rate of PXO61 on transgenic plants was significantly higher (\( P < 0.05 \)) than the growth rate on wild-type plants at 8–12 days after infection (Figure 2b). These results suggest that the increased susceptibility of the transgenic plants may be attributable to the suppression of OsPAD4.

To examine this hypothesis, T\(_1\) plants of two T\(_1\) families generated from two susceptible T\(_0\) plants (D146RM6 and D146RM7) were further analyzed individually at the booting stage for their response to the Xoo strain PXO99 and the OsPAD4 transcript level. The increased susceptibility of T\(_1\) plants co-segregated with reduced OsPAD4 transcripts (Figure 2c). The correlations between disease area and OsPAD4 transcripts were \(-0.5262\) (significant at \( \alpha = 0.05; n = 15 \)) and \(-0.5724\) (significant at \( \alpha = 0.05; n = 15 \)) for D146RM6 and D146RM7 T\(_1\) families, respectively. T\(_2\) plants of two T\(_2\) families from the two T\(_1\) families were also analyzed individually at the booting stage for their response to the Xoo strain PXO61 and OsPAD4 expression. The increased susceptibility of these T\(_2\) plants negatively correlated with OsPAD4 transcript levels (D146RM6-7 T\(_2\) family, \( r = -0.8312\); significant at \( \alpha = 0.01; n = 14 \); D146RM7-3 T\(_2\) family, \( r = -0.5464\); \( \alpha = 0.05; n = 15 \); Figure S3). These results confirm that the increased susceptibility of transgenic plants is associated with the suppression of OsPAD4.

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**Figure 1.** Phylogenetic analysis of the evolution of PAD4-type proteins and subcellular localization of OsPAD4.

(a) PAD4-type proteins are evolutionarily conserved in different plant species. Sequences were analyzed by the unweighted pair group method using the arithmetic method, with genetic distance calculated by \( \text{neighbour} \) 5.2 (http://www.megasoftware.net). The numbers for interior branches indicate the bootstrap values (%) for 1000 replications. The scale bar represents 0.5 amino acid substitutions per site. The accession numbers of some PAD4-like proteins in the NCBI protein database (http://www.ncbi.nlm.nih.gov) are NP_001067424 (Oryza sativa, rice), XP_002449170 (Sorghum), NP_001242860/XP_003532051 (Glycine max, soybean), and XP_00222923 (Ricinus communis, castor bean), XP_003577748 (Brachyphyllum distachyon), XP_002532970 (Populus, poplar), XP_001248606/XP_003532051 (Glycine max, soybean), and CB12592 (Vitis vinifera, wine grape). The accession numbers of other PAD4-like proteins are evm.model superstar_37.13 (Carica papaya, papaya), Cucsa.353810.1 (Cucumis sativus, cucumber), BAJ89353 (Hordeum vulgare, barley), cassava4.1.004098m (Manihot esculenta, cassava), mgv1a004394m (Mimulus guttatus, spotted monkey flower), and Si026081m (Setaria italica, foxtail millet) in PlantGDB (http://www.plantgdb.org/prj/Genome-Browser).

(b) OsPAD4-GFP fusion protein localized in the plasma membrane of rice protoplasts. The \( P_{35S}:OsPAD4-GFP \) was transiently expressed in stem rice protoplasts isolated from japonica rice variety Zhonghua 11. Bars represent 10 \( \mu \)m.
is a positive regulator in the resistance of rice to Xoo. We refer to these transgenic plants as Os\textit{PAD4} RNAi plants.

**Suppressing Os\textit{PAD4} did not influence gene-for-gene resistance to Xoo**

\textit{AtPAD4} is required for \textit{RPS4}-mediated and \textit{RPP5}-mediated gene-for-gene resistance to \textit{Pseudomonas syringae} and \textit{Phytophthora parasitica} in Arabidopsis (Glazebrook et al., 1997; Parker et al., 2000). \textit{RPS4} and \textit{RPP5} encode cytoplasmic nucleotide-binding leucine-rich repeat (LRR) proteins (Leister et al., 1996; Parker et al., 1997). To ascertain whether suppressing \textit{OsPAD4} influenced \textit{MR} gene-mediated race-specific resistance, we crossed an \textit{OsPAD4}-RNAi plant (D146RM7) with transgenic rice line Rb49, which carries \textit{MR} gene \textit{Xa3}/\textit{Xa26} and has the genetic background of Mudanjiang 8. \textit{Xa3}/\textit{Xa26}, encoding an LRR receptor kinase-like protein, confers race-specific resistance to Xoo, and rice plants carrying \textit{Xa3}/\textit{Xa26} are resistant to Xoo strain PXO61 but are susceptible to Xoo strain PXO99 (Sun et al., 2004; Cao et al., 2007). The \textit{F}$_2$ plants (Rb49/Os\textit{PAD4}-RNAi) carrying both Os\textit{PAD4}-RNAi construct and \textit{Xa3}/\textit{Xa26} generated from this cross were analyzed. These \textit{F}$_2$ plants showed a level of resistance to PXO61 similar to that against Rb49, but they were more susceptible to PXO99 than to Rb49 (Figure 3a). These results suggest that \textit{OsPAD4} may not function in \textit{Xa3}/\textit{Xa26}-initiated defense signaling.

![Figure 2](https://example.com/fig2.png)

Figure 2. Os\textit{PAD4}-transgenic rice plants (D146RM) had increased susceptibility to Xoo. Plants were inoculated with Xoo at the booting stage. Data represent means (three replicates from one plant for gene expression, between two and five replicates from one plant for disease area, and three replicates from each type of plant for bacterial growth) ± standard deviations. The ‘a’ and ‘b’ indicate a significant difference was detected between transgenic plants and wild-type (WT; Mudanjiang 8) plants ($P < 0.01$ or $P < 0.05$, respectively).

(a) Transgenic T$_0$ plants with suppressed Os\textit{PAD4} expression showed increased susceptibility to Xoo strain PXO61. D146RM8 is a negative transgenic plant.

(b) Growth of Xoo strain PXO61 in the leaves of Os\textit{PAD4}-suppressing T$_2$ (D146RM6-7 and D146RM7-3) plants; cfu, colony-forming units.

(c) Increased susceptibility of Os\textit{PAD4}-suppressing T$_1$ plants to Xoo strain PXO99 was associated with suppressed Os\textit{PAD4} expression.
To further examine the inference that OsPAD4 does not function downstream of Xa3/Xa26 in defense signaling, we crossed the OsPAD4-RNAi line (D146RM7) with the WRKY13-overexpressing (WRKY13-oe) transgenic rice line (D11UM7-2), which has the genetic background of Mudanjiang 8 (Qiu et al., 2007). WRKY13 is a transcriptional repressor that positively regulates the quantitative resistance of rice to both Xoo and M. oryzae, and negatively regulates the responses of rice to abiotic stresses. Suppressing WRKY13 compromises Xa3/Xa26-mediated resistance against Xoo, indicating that WRKY13 functions in the defense signaling pathway downstream of Xa3/ Xa26 (Qiu et al., 2007, 2008, 2009; Xiao et al., 2013). The F2 plants (OsPAD4-RNAi7/WRKY13-oe) from this cross were analyzed. The F2 plants carrying both OsPAD4-RNAi and WRKY13-oe constructs showed increased resistance to Xoo compared with the F2 plants carrying only the OsPAD4-RNAi construct and the OsPAD4-RNAi line, but they showed increased susceptibility to Xoo compared with the F2 plants carrying only the WRKY13-oe construct and the WRKY13-oe line (Figure 3b). These results suggest that OsPAD4 and WRKY13 do not function in the same defense signaling pathway as in the rice-Xoo interaction, further supporting the inference that OsPAD4 is not involved in Xa3/Xa26-mediated resistance.

**Figure 3.** Analyses of the relationship of OsPAD4 and MR gene-mediated resistance to Xoo. Rice variety Mudanjiang 8 is a wild type (WT) and is susceptible to Xoo strains PXO61 and PXO99. Rb49 and D49OM6 are transgenic lines carrying transgenic MR genes Xa3/Xa26 and Xa21, respectively, driven by their native promoters and with the genetic background of Mudanjiang 8; D146RM7 (M7), OsPAD4-RNAi transgenic line with the genetic background of Mudanjiang 8; WRKY13-oe, WRKY13-overexpressing line D11UM7-2 with the genetic background of Mudanjiang 8. Plants were inoculated with Xoo strain PXO61 or PXO99 at the booting stage. Gene expression was analyzed by qRT-PCR. Bars represent means (three replicates for gene expression and 10-25 leaves from two and five plants for disease area) ± standard deviation. The ‘a’ and ‘b’ indicate that a significant difference was detected between inoculated plants and non-inoculated control (ck) plants (P < 0.01 or P < 0.05, respectively). Asterisks indicate that a significant difference was detected between wild-type plants and other plants after the same treatment (**P < 0.01 and *P < 0.05).

(a) OsPAD4 was not involved in Xa3/Xa26-mediated resistance. F2 plants carrying both OsPAD4-RNAi construct and Xa3/Xa26 were generated from a cross between Rb49 and D146RM7.

(b) OsPAD4 and WRKY13 did not function in the same defense signaling pathway during the rice-Xoo interaction. F2 plants were generated from the cross between D146RM7 and WRKY13-oe lines.

(c) OsPAD4 showed differential expression in resistant and susceptible reactions after infection with Xoo.

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These results are consistent with differential expression patterns of OsPAD4 in resistant and susceptible reactions (Figure 3c). Rice variety Mudanjiang 8, which is susceptible to PXO61, is the wild type for transgenic rice lines Rb49 and D49OM6 (Sun et al., 2004; Cao et al., 2007; Zhao et al., 2009). D49OM6 carries the MR gene Xa21, which encodes LRR receptor kinase protein and also mediates race-specific resistance to Xoo (Zhao et al., 2009). OsPAD4 expression in Rb49 and D49OM6 showed a similar pattern in comparison with OsPAD4 expression in wild-type plants. OsPAD4 expression was induced to a maximum level at 8 h after infection in both resistant and susceptible reactions (Figure 3c); however, the OsPAD4 transcript levels were significantly higher in susceptible wild-type plants compared with the resistant Rb49 and D49OM6 plants before infection, and during most time points after infection. These results suggest that OsPAD4 may not be specific for Xa21-mediated resistance either, although it is involved in the regulation of the rice-Xoo interaction.

Wound-induced systemic resistance was impaired in OsPAD4-RNAi plants

AtPAD4 is required for SAR establishment (Jing et al., 2011; Shah and Zeier, 2013). To determine whether OsPAD4 is involved in rice SAR, we first inoculated the leaves of rice plants that developed early with Xoc strain RH3, and then inoculated the leaves on the same tiller that developed later with Xoo strain PXO99 at 1, 2 or 3 days after the first inoculation. OsPAD4-RNAi plants were more susceptible to RH3 compared with wild-type plants (Figure 4a). After the first inoculation with RH3, OsPAD4-RNAi and wild-type plants, as well as the negative siblings (OsPAD4-RNAi–) from OsPAD4-RNAi segregating populations, showed less susceptibility to Xoo compared with the plants without the first inoculation (Figure 4b); however, suppressing OsPAD4 impaired the induced systemic resistance. In the systemic leaves, the disease area of wild-type/OsPAD4-RNAi– plants caused by Xoo infection was reduced by more than 50%, whereas the disease area of OsPAD4-RNAi plants caused by Xoo was only reduced by approximately 20%. Mock inoculation with phosphate-buffered saline (PBS) also reduced the susceptibility of OsPAD4-RNAi and wild-type/OsPAD4-RNAi– plants to Xoo in systemic leaves (Figures 4b and S4). This reduced susceptibility was also impaired after suppressing OsPAD4. The OsPAD4-RNAi plants had fewer reduced disease areas and higher bacterial growth rates compared with those of wild-type/OsPAD4-RNAi– plants in systemic tissue after mock inoculation (Figures 4b and S4). In addition, inoculation and mock inoculation resulted in a similar level of reduced susceptibility to Xoo in systemic leaves of both OsPAD4-RNAi and wild-type plants. Inoculation with Xoc and mock inoculation caused wounds. These results suggest that OsPAD4 may be required for wound-induced systemic resistance instead of SAR.

Figure 4. The responses of OsPAD4-RNAi plants to bacterial infection and wounding; WT, wild-type Mudanjiang 8; 6+ and 7+, OsPAD4-RNAi T2 lines D146RM6 and D146RM7, respectively; 6– and 7–, negative siblings from D146RM6 and D146RM7 segregating populations, respectively. Bars represent means (between five and 12 leaves from between two and four plants for disease area and three replicates for gene expression) ± standard deviations. The ‘a’ and ‘b’ indicate a significant difference was detected between wild-type and OsPAD4-RNAi plants or between treated and untreated control (ck) plants (P < 0.01 or P = 0.05, respectively; a and c). (a) Suppressing OsPAD4 increased rice susceptibility to Xoc strain RH3 as shown at 15-17 days after inoculation. (b) Suppressing OsPAD4 compromised induced resistance to Xoo in rice. Plants were first inoculated in the early-developing leaves with Xoc strain RH3 or PBS (mock inoculation), or without inoculation (none), and were then inoculated in the late-developing leaves with Xoo strain PXO61 at 1-3 days after the first inoculation. Asterisks indicate that a significant difference was detected between no first treatment (none) and treatment within the same plant material (**P < 0.01 and *P < 0.05). (c) Wounding induced OsPAD4 in both local and unwounded systemic leaves in rice variety Mudanjiang 8, as analyzed by qRT-PCR.
To further determine whether OsPAD4 was essential for wound-induced systemic resistance, we analyzed OsPAD4 expression after wounding by mock inoculation with PBS. OsPAD4 expression was rapidly induced in local (early) leaves at 1 h, and reached the highest level at 12 h after wounding (Figure 4c). Its expression was also rapidly induced in late-developing unwounded leaves at 1 h, and reached the highest level at 3 h after wounding the early-developing leaves (Figure 4c). This result suggests that OsPAD4 may play a role in wound-induced bacterial resistance.

Impaired disease resistance was associated with repressed JA signaling in OsPAD4-RNAi plants

Jasmonic acid (JA) is a wound-responsive hormone, and wounding can increase the accumulation of JA (Mousavi et al., 2013). To determine whether OsPAD4-mediated resistance was JA dependent, we examined the expression of JA-responsive genes. Rice PR1a expression was markedly induced by wounding, JA and SA, but JA and SA only weakly influenced OsPAD4 expression (Figure S5; Agrawal et al., 2000). AOS2 encodes allene oxide synthase, a key enzyme in the JA biosynthetic pathway in rice (Mei et al., 2006). Rice JAZ8, encoding the jasmonate ZIM-domain (JAZ) protein, is induced by pathogens and JA, and functions in JA-dependent signaling (Figure S5; Liu et al., 2012; Yamada et al., 2012). The expression of JAZ8, PR1a and AOS2 was induced in infected rice leaves after inoculation with Xoo and in unwounded systemic leaves after wounding in wild-type and OsPAD4-RNAi plants (Figure 5). The transcript levels of the three genes in OsPAD4-RNAi plants were significantly lower than those in wild-type plants without infection, however, and after Xoo infection. Consistent with the expression patterns of these genes, the JA content was increased in infected rice leaves and in unwounded systemic leaves, but the JA content in OsPAD4-RNAi plants was significantly lower than that in wild-type plants (Figure 5).

Arabidopsis AtPAD4-mediated defense responses are SA dependent (Zhou et al., 1998). To ascertain whether OsPAD4-mediated bacterial resistance was associated with SA, we analyzed the SA content in the same samples used for JA quantification and JA-related gene expression analyses (Figure 5). The SA content was reduced but there

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**Figure 5.** Suppressing OsPAD4 markedly reduced the accumulation of jasmonic acid (JA) and the phytoalexin monilactone A (MOA), and the expression of JA-responsive genes and the JA synthesis-related gene. Gene expression was analyzed by qRT-PCR. Bars represent means (from three replicates) ± standard deviations. The ‘a’ and ‘b’ indicate a significant difference was detected between treated and untreated control (ck) plants (P < 0.01 or P < 0.05, respectively). Asterisks indicate that a significant difference was detected between wild-type plants and OsPAD4-RNAi plants after the same treatment (***P < 0.01 and **P < 0.05). FW, fresh weight.
Exogenous application of JA reduced the growth of bacterium; 0 day, 1 h after inoculation of rice to Xoo (Yamada et al., 2012). Consistent with previous reports, exogenous application of JA reduced rice susceptibility to Xoo (Figure 6a). JA treatment complemented the phenotype of OsPAD4-RNAi plants: these plants showed disease areas and bacterial growth rates similar to those of mock-treated wild-type plants ($P > 0.05$; Figures 6a, b). Suppression of OsPAD4 impaired the enhanced resistance to Xoo conferred by exogenous JA, however: the OsPAD4-RNAi plants had fewer reduced disease areas and higher bacterial growth rates compared with those of JA-treated wild-type plants.

Exogenous application of SA can induce rice resistance to both Xoo and the fungal pathogen M. oryza (Song et al., 2001; Iwai et al., 2007; Xu et al., 2013). To ascertain whether the increased susceptibility of OsPAD4-RNAi plants could be complemented by the exogenous application of SA, we treated rice plants with either 200 $\mu$M or 5 $\mu$M of SA. Both treatments could not induce rice resistance to Xoo (Figure S5), although exogenous application of SA (200 $\mu$M) was able to induce the expression of $PR1a$ (Figure S5). These results further support the inference that OsPAD4 functions in the JA-dependent pathway in the interaction between rice and Xoo.

**Accumulation of phytoalexins was impaired in OsPAD4-RNAi plants**

Arabidopsis AtPAD4 positively regulated the pathogen-induced accumulation of indole-type phytoalexin camalexin (Zhou et al., 1998). To test whether Xoo induced rice phytoalexin accumulation dependent on OsPAD4, we quantified the known rice phytoalexins, the terpenoid-type phytoalexin momilactone A (MOA) and the flavonoid-type phytoalexin sakuranetin, in OsPAD4-RNAi plants after Xoo infection. Xoo infection induced MOA accumulation in wild-type plants, and MOA also rapidly accumulated in unwounded systemic leaves of wild-type plants after wounding (Figure 5). However, the MOA content in the local infected leaves and systemic leaves after wounding of OsPAD4-RNAi plants was significantly lower than that in wild-type plants (Figure 5). In contrast, the sakuranetin content was very low (fresh weight approximately 0.005–0.05 $\text{ng g}^{-1}$) in wild-type and OsPAD4-RNAi plants. These results suggest that the increased susceptibility of OsPAD4-RNAi plants may also be associated with the reduced accumulation of MOA.

**DISCUSSION**

PAD4 is a putative triacylglycerol lipase. The role of Arabidopsis AtPAD4 in pathogen-induced defense responses has been studied extensively. PAD4 is involved in the modulation of SA accumulation and the induction of defense responses in Arabidopsis. The JA and SA pathways interact in the regulation of defense responses, with JA being a key activator of SA accumulation.

![Figure 6](image_url)

**Figure 6.** Exogenous application of jasmonic acid (JA) influenced *Oryza sativa* (rice) response to *Xoo* invasion in wild-type (WT) and OsPAD4-RNAi plants. Plants were sprayed with 150 $\mu$M JA or a solution not containing JA (mock) during the tillering to booting stages; 2 days after treatment, plants were inoculated with *Xoo* strain PX061. Bars represent means (17–44 replicates from between eight and 16 plants for disease area and three replicates for bacterial growth) ± standard deviations. The ‘a’ indicates a significant difference was detected between the same plants with JA treatment and mock treatment ($P < 0.01$). Asterisks (**) indicate that a significant difference was detected between WT plants and OsPAD4-RNAi plants after the same treatment ($P < 0.001$).

(a) Exogenous application of JA reduced rice susceptibility to Xoo.
(b) Exogenous application of JA reduced the growth of bacterium; 0 day, 1 h after inoculation of Xoo; cfu, colony-forming units.
been extensively studied. AtPAD4 functions as a positive regulator in the SA-dependent signaling pathway, and as a negative regulator in the JA-dependent signaling pathway, in disease resistance of Arabidopsis (Zhou et al., 1998; Brodersen et al., 2006). Rice OsPAD4 is the sequence ortholog of AtPAD4. The present results suggest that OsPAD4 positively regulates rice defense responses to bacterial pathogens: suppressing OsPAD4 compromises the resistance of rice to Xoo and Xoc. The following evidence suggests that OsPAD4 may not be the functional ortholog of AtPAD4 in host-pathogen interactions, however, considering that OsPAD4 is a plasma membrane protein and that AtPAD4 is a cytoplasmic and nuclear protein. OsPAD4 and AtPAD4 appear to play different roles in host resistance against pathogens.

**OsPAD4 is required for wound-induced systemic resistance**

Induced immunity is an important method used by plants to defeat pathogen attacks. Wounding allows pathogens, including Xoo and Xoc, to invade plants (Li and Wang, 2013). Plants can also be wounded by the invasion of some pathogens (Balmer et al., 2013; Kobayashi and Kobayashi, 2013). Wound-induced resistance is a strategy used by plants to defend themselves from pathogen challenges. Wounding can induce disease resistance in dicots and monocots (Schweizer et al., 1998; Walters et al., 2006). AtPAD4 mediates SA-dependent SAR to biotrophic pathogens, but not wound-induced systemic resistance in Arabidopsis (Jing et al., 2011; Rietz et al., 2011). The present results show that OsPAD4 is involved in induced resistance to Xoo. Suppressing OsPAD4 impaired induced disease resistance; however, this gene is required for wound-induced systemic resistance and may not be essential for SAR, and is supported by the following evidence. A first infection and a mock (wound) infection induced the same level of impaired resistance to Xoo in systemic tissues of OsPAD4-RNAi plants (Figure 4b). Wounding stimulates JA biosynthesis (Chung et al., 2008; Glauser et al., 2008; Koo et al., 2009). The increased susceptibility of OsPAD4-RNAi plants to Xoo was associated with reduced JA content in local tissues, and the wound-induced accumulation of JA in systemic tissues was compromised in OsPAD4-RNAi plants (Figure 5). Finally, OsPAD4 expression was rapidly induced not only in wounded tissues but also in unwounded systemic tissues (Figure 4c). These results suggest that OsPAD4 and AtPAD4 play different roles in induced resistance: OsPAD4 is required for wound-induced resistance and AtPAD4 is required for SAR.

**OsPAD4-involved defense signaling is JA dependent**

In general, resistance against biotrophic and hemibiotrophic pathogens is usually regulated by the SA-dependent pathway, whereas resistance against necrotrophic pathogens is frequently controlled by the JA/ethylene-dependent pathway (Bari and Jones, 2009). AtPAD4 as a positive regulator modulates Arabidopsis resistance to biotic pathogens in an SA-dependent manner, but as a negative regulator it mediates Arabidopsis resistance to necrotrophic pathogen in a JA-dependent pathway (Glazebrook et al., 1997; Zhou et al., 1998; Brodersen et al., 2006). Xoo is a biotrophic bacterium (Kou and Wang, 2013). These results show that suppressing OsPAD4 compromised Xoo-induced JA accumulation and JAZ8 expression, which is a marker of JA-dependent signaling (Yamada et al., 2012), in local tissues and wound-induced JA accumulation in unwounded systemic tissues (Figure 5). Exogenous application of JA complemented the phenotype of OsPAD4-RNAi plants in response to Xoo infection, however (Figure 6a). These results suggest that unlike AtPAD4, OsPAD4 plays a role in JA-dependent signaling in rice resistance against biotrophic pathogens.

**Accumulation of MOA may contribute to the OsPAD4-mediated defense response**

One of the plant defense responses against pathogens is producing secondary metabolites, such as phytoalexins, to kill pathogens (Ahuja et al., 2012). Until now, only two types of phytoalexins, camalexin and rapalexin A, have been detected in Arabidopsis (Ahuja et al., 2012). AtPAD4 functions in the SA-dependent pathway and positively regulates pathogen-induced phytoalexin camalexin accumulation in Arabidopsis-pathogen interactions (Zhou et al., 1998). The indole-type camalexin, which is synthesized from tryptophan by a cytosolic pathway, is the major phytoalexin form in Arabidopsis (Glawischnig, 2007; Geu-Flores et al., 2011). It is suggested that JA signaling controls camalexin synthesis (Rowe et al., 2010); however, SA signaling-independent camalexin synthesis and signaling-dependent camalexin synthesis have also been proposed by different studies (Nawrath and Metraux, 1999; Roetschi et al., 2001; Denby et al., 2005).

The synthesis of camalexin in rice has not yet been reported; however, rice can synthesize the terpenoid phytoalexin MOA, which has not been reported in Arabidopsis. MOA accumulation was induced by M. oryzae in rice (Hasegawa et al., 2010; Riemann et al., 2013). MOA can inhibit the growth of the rice fungal pathogen M. oryzae (Dillon et al., 1997; Grayer and Kokubun, 2001; Hasegawa et al., 2010). In addition, Xoo infection also induced MOA accumulation in rice (Liu et al., 2012). Exogenous JA induced MOA accumulation (Rakwal et al., 1996), and M. oryzae-induced MOA accumulation was suppressed in the JA-deficient mutant (Riemann et al., 2013). These results suggest that MOA accumulation is dependent on JA signaling. Our present results also show that Xoo infection induces the accumulation of MOA in wild-type plants, and that reduced MOA content is associated with reduced
JA levels in OsPAD4-RNAi plants, indicating that the impaired resistance of OsPAD4-RNAi plants may be at least partly attributable to the reduced accumulation of MOA. Thus, OsPAD4 functions differently from AtPAD4 by promoting MOA synthesis in a JA-dependent pathway.

In conclusion, OsPAD4 functions differently than AtPAD4 in response to pathogen infection. This difference may arise because OsPAD4 is a plasma membrane-associated protein and AtPAD4 is a cytoplasmic and nuclear protein. Future studies with the subcellular localization domain switch may provide insight on this perspective.

EXPERIMENTAL PROCEDURES

Bioinformatics

The amino acid sequence of Arabidopsis AtPAD4 (accession number NP_190811 in the protein database of the National Center for Biotechnology Information, NCBI; http://www.ncbi.nlm.nih.gov) was used to screen the rice genome database (http://blast.ncbi.nlm.nih.gov/Blast) using the BLASTP program (Altschul et al., 1997). The rice amino acid sequence NP_001067424 (gene locus LOC_Os11g09010) showed the highest sequence similarity to AtPAD4. The genomic sequence of LOC_Os11g09010 was used to search the Knowledge-based Oryza Molecular Biological Encyclopedia database [KOME; http://cdna01.dna.affrc.go.jp/cDNA/et al.], and a full-length cDNA AK234523 (cDNA clone J100075L24) corresponding to LOC_Os11g09010 from japonica rice (O. sativa ssp. japonica) variety Nipponbare was identified. This cDNA clone was kindly provided by the RIKEN Yokohama Institute (Suzuki et al., 1997).

Web-based topology prediction programs TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), HMMTOP (http://www.enzim.hu/hmmtop), and PHOBIUS (http://phobius.sbc.su.se/) were used to predict the orientation of the transmembrane regions of PAD4 protein.

Subcellular localization of OsPAD4

To determine the subcellular localization of the OsPAD4 protein, the OsPAD4 gene was fused with the green fluorescent protein (GFP) gene at the 3' end. The coding region of OsPAD4, amplified from cDNA clone J100075L24 using gene-specific primers (Table S1), was cloned into vector pU1391 for the stable expression of OsPAD4-GFP. The pU1391 vector was constructed by inserting a 566-bp DNA fragment containing the maize ubiquitin promoter (PUbi) and GFP into vector pCOMBIA1391 (Shen et al., 2010). For transient expres-

sion of OsPAD4-GFP, the coding region of OsPAD4 was cloned into the pM999-GFP vector to produce the OsPAD4-GFP fusion construct driven by a cauliflower mosaic virus 35S promoter (PUbi). The construct was transiently expressed in rice stem protoplasts, as described by Zhang et al. (2011). Green fluorescent signal was examined using a confocal microscope (TCS SP2; Leica, http://www.leica.com).

Rice transformation

To construct an RNAi vector of OsPAD4, a 566-bp DNA fragment amplified from OsPAD4 cDNA using gene-specific primers (Table S1) was inserted into transformation vector pDS1301 (Yuan et al., 2007). The OsPAD4-RNAi and OsPAD4-GFP vectors were transformed into Agrobacterium tumefaciens strain EHA105 by electro-

poration. Agrobacterium-mediated transformation was performed using calli derived from mature embryos of japonica rice varieties Mudanjiang 8 or Zhonghua 11 (Lin and Zhang, 2005).

Wound treatment

The leaves of rice plants at the booting (panicle development) stage were penetrated with 500 μl of 10 mM PBS using a syringe (Schaad et al., 1996).

Pathogen inoculation

To evaluate bacterial blight disease, transgenic and wild-type plants were inoculated with Philippine Xoo strains PXO61 and PXO99 by the leaf-clipping method during the booting stage (Chen et al., 2002). The extent of disease was scored by measuring the percentage of disease area [(lesion length/leaf length) × 100] at approximately 10 days after inoculation. The bacterial growth rate in rice leaves was measured by counting the colony-forming units (Sun et al., 2004).

For analysis of systemic acquired resistance, the early-developing leaves of rice plants were infected with Chinese Xoc strain RH3 by the penetration method using a syringe, and then the late-
developing leaves on the same tiller were inoculated with PXO99 at 1-3 days after the first inoculation. The Xoc inoculum was pre-

pared using 10 mM of PBS.

For analysis of wound-induced systemic resistance, the early-
developing leaves of rice plants were wounded and then the late-
developing leaves on the same tiller were inoculated with PXO99 at 3 days after wounding.

Hormone treatment

Rice plants growing in a glasshouse until the booting stage were sprayed with 150 μM JA, or with 200 μM or 5 mM SA, in 0.1% (v/v) methanol and 0.015% (v/v) Tween 20, or were mock-sprayed with 0.1% methanol and 0.015% Tween 20 until uniformly wet. The sprayed plants were kept sealed in plastic shade for 2 days. After hormone treatment, the plants were inoculated with Xoo strain PXO61.

Gene expression analysis and quantification of SA and JA

The 3-cm leaf fragments next to the bacterial infection sites were used for RNA isolation and phytohormone quantification. Quantita-

tive reverse-transcription polymerase chain reaction (qRT-PCR) was conducted using gene-specific primers (Table S2), as described previously (Qiu et al., 2007). The expression level of the rice actin gene was used to standardize the RNA sample for each qRT-PCR. The expression level relative to that of controls was presented.

The same samples used for gene expression analysis were used for phytohormone quantification. Samples were prepared and JA and SA were quantified using an ultrafast liquid chromatograph/ electrospray ionization/tandem mass spectrometry system, as described previously (Liu et al., 2012).

Statistical analyses

The significant differences between control samples and treated samples were analyzed by pairwise Student’s t-tests in EXCEL (Microsoft, http://www.microsoftstore.com). The correlation analy-

sis between gene transcript level and disease level was performed using CORREL analysis in EXCEL.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Alignment of the lipase regions of PAD4 homologs from different species.

Figure S2. OsPAD4-GFP fusion protein localized in the plasma membrane of rice protoplasts.

Figure S3. An increased susceptibility of OsPAD4-suppressing T2 plants to Xoo strain PXO61 was associated with suppressed OsPAD4 expression.

Figure S4. Suppressing OsPAD4 compromised induced resistance to Xoo in rice.

Figure S5. Exogenous application of JA and SA markedly influenced the expression of PR1a and JAZ2 analyzed by qRT-PCR.

Figure S6. Exogenous application of SA did not influence the rice response to Xoo in both wild-type (WT) and OsPAD4-RNAi plants.

Table S1. PCR primers used for the construction of vectors, detection of positive transgenic plants and sequencing.

Table S2. Primers used for quantitative PCR in gene expression analysis.

REFERENCES


encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl Acad. Sci. USA, 96, 13583–13588.


