Two Different Transcripts of a LAMMER Kinase Gene Play Opposite Roles in Disease Resistance[1[OPEN]]

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Alternative splicing of genes can increase protein diversity and affect mRNA stability. Genome-wide transcriptome sequencing has demonstrated that alternative splicing occurs in a large number of intron-containing genes of different species. However, despite the phenomenon having been known for decades, it is largely unknown how the alternatively spliced transcripts function differently. Here, we report that two alternatively spliced transcripts of the rice (*Oryza sativa*) LAMMER kinase gene *OsDR11*, long *OsDR11L* and short *OsDR11S*, play opposite roles in rice resistance against *Xanthomonas oryzae* pv *oryzae* (*Xoo*), which causes the most damaging bacterial disease in rice worldwide. Overexpressing *OsDR11S* or suppressing *OsDR11L* in rice enhanced resistance to *Xoo*, which was accompanied by an accumulation of jasmonic acid (JA) and induced expression of JA signaling genes. In contrast, suppressing *OsDR11S* was associated with increased susceptibility to *Xoo*, along with decreased levels of JA and expression of JA signaling genes. The OsDR11S and OsDR11L proteins colocalized in the nucleus. OsDR11L showed autophosphorylation activity in vitro, while OsDR11S did not. In the presence of OsDR11S, autophosphorylation of OsDR11L was inhibited, and overexpression of *OsDR11S* suppressed *OsDR11L* expression. *OsDR11* appeared to contribute to a minor quantitative trait locus against *Xoo*. These results suggest that OsDR11L is a negative regulator in rice disease resistance, which may be associated with suppression of JA signaling. The results also suggest that OsDR11S may inhibit the function of OsDR11L at both the transcription and protein kinase activity levels, leading to resistance against *Xoo*.

Bacterial blight caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is the most damaging bacterial disease in rice (*Oryza sativa*) worldwide. Resistance to *Xoo* can be classified into two main genetic categories in rice: qualitative resistance regulated by major disease resistance (*MR*) genes and quantitative resistance regulated by multiple genes or quantitative trait loci (QTLs; Kou and Wang, 2010; Zhang and Wang, 2013). An *MR* gene against *Xoo* may be a disease-resistance gene that initiates effector-triggered immunity, a pattern-recognition receptor gene that initiates pathogen-associated molecular pattern-triggered immunity, or a gene that mediates resistance in a manner that cannot be explained by present immunity models (Zhang and Wang, 2013). Several resistance QTLs contributed by defense-responsive genes that function downstream of *MR*-initiated defense signaling pathways against *Xoo* have been identified (Kou and Wang, 2012, 2013). One important group of rice defense-responsive genes encode protein kinases that participate in rice-*Xoo* interactions (Yuan et al., 2007; Shen et al., 2010, 2011; Geng et al., 2013; Chen et al., 2014).

The LAMMER kinases, named after the EHLAMMERILG motif that is important for kinase activity, are conserved in eukaryotes. These kinases have been reported to be involved in various biological activities and physiological processes, including development, cell differentiation, abiotic stress response, metabolism, and reproduction in yeast, insect, animal, and human (Yun et al., 1994; García-Sacristán et al., 2005; James et al., 2009; Rabinow and Samson, 2010; Rodgers et al., 2010). One of the best known biochemical functions of LAMMER kinases is the regulation of alternative splicing via phosphorylation of Ser/Arg-rich (SR) splicing factors and SR-like proteins or other substrates such as cyclin-dependent kinase-inhibitor Rum1 (Kang et al., 2013; Yu et al., 2013; Zhao et al., 2013).

To date, the LAMMER kinases have only been studied in Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*). The tobacco LAMMER kinase PK12 is induced by phytohormone ethylene in vivo, and it phosphorylates both animal and plant proteins in vitro (Sessa et al., 1996; Savaldi-Goldstein

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et al., 2000). In Arabidopsis, overexpression of *PK12* influences development (Savaldi-Goldstein et al., 2003). Arabidopsis has three members of the LAMMER kinase family (Savaldi-Goldstein et al., 2003). AFC1 can complement a yeast mitogen-activated protein kinase mutant (Bender and Fink, 1994). AFC2, but not AFC1, has the ability to phosphorylate SR splicing factors (Golovkin and Reddy, 1999; Reddy and Shad Ali 2011); however, it is unknown whether plant LAMMER kinases are involved in the regulation of host-pathogen interactions.

Alternative splicing of a gene is a widespread post-transcriptional regulatory mechanism that can increase protein diversity and affect mRNA stability. Genome-wide high-throughput transcriptome sequencing demonstrated that alternative splicing occurs in 95% of intron-containing genes in human and 61% of those in Arabidopsis (Pan et al., 2008; Marquez et al., 2012). Extensive alternative splicing events also occur in rice, with 33% to 48% of intron-containing genes being involved (Lu et al., 2010; Zhang et al., 2010). Despite the phenomenon of alternative splicing having been known for decades, however, the biological roles of alternatively spliced transcripts from a single gene are poorly understood.

A previous study revealed that rice cDNA clone EI39C8 (GenBank accession no. BF108323), which corresponds to *OsDR11* and encodes a LAMMER kinase-type protein, is a pathogen-induced defense-responsive gene (Zhou et al., 2002). To evaluate whether *OsDR11* is involved in rice disease resistance, we generated *OsDR11*-transgenic plants and analyzed the kinase activity of OsDR11. The results suggest that *OsDR11* can generate two types of transcripts, the long *OsDR11L* and the short *OsDR11S*, which are differentially expressed in rice-*Xoo* interaction. OsDR11L has protein kinase activity and suppresses rice disease resistance; OsDR11S does not possess kinase activity and promotes resistance. The ability of OsDR11L to undergo autophosphorylation is influenced by the presence of OsDR11S in vitro. *OsDR11* may contribute to a minor *Xoo*-resistance QTL, which may operate through OsDR11S inhibition of OsDR11L function.

RESULTS

Transcriptional and Structural Characterization of *OsDR11*

Previous study showed that pathogen infection induced expression of full-length cDNA EI39C8 of *OsDR11* from rice variety Minghui 63 (Zhou et al., 2002). EI39C8 is one of the alternatively spliced transcripts of the Os12g27520 locus, according to the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). It contains 1518 nucleotides (nt) and encodes a protein consisting of 170 amino acids. Based on DNA gel blot analysis and genome-wide sequence analysis, Minghui 63, rice variety Zhenshan 97, and genome-sequenced rice variety Nipponbare all harbored a single copy of *OsDR11* (Supplemental Fig. S1). We found that the Minghui 63 cDNA library (Chu et al., 2003) also contained another full-length cDNA, EI114F3, from *OsDR11*. EI114F3 has 1496 nt and encodes a protein consisting of 370 amino acids. In the following text, we refer to EI39C8 and EI114F3 as *OsDR11S* and *OsDR11L*, respectively (Fig. 1A). *OsDR11L* has 11 exons and the translation terminates at exon 10, while *OsDR11S* has six exons and excludes exons 5, 6, and 7, resulting in a shift in the reading frame and leading to premature termination of translation (Fig. 1A). Both alternative transcripts *OsDR11S* and *OsDR11L* were also found in rice varieties Mudanjiang 8 and Zhenshan 97.

The products encoded by *OsDR11* were highly homologous with the defined LAMMER kinases. Sequence analysis showed that *OsDR11L* encodes a LAMMER kinase-type protein based on the conserved LAMMER motif EHLAMMERVFG (Fig. 1B). However, the protein deduced from *OsDR11S* does not include the entire LAMMER protein kinase domain because of the premature termination of translation. Alternative splicing of LAMMER kinases also exist in other species, such as protein kinase Clk/Sty in mammalian and AFC2 in Arabidopsis, which leads to truncated proteins lacking the LAMMER motif, like OsDR11S (Duncan et al., 1997; Marquez et al., 2012). Sequence comparison of the full-length forms showed that OsDR11L shares 41% to 71% identity with other LAMMER kinases from various organisms. The LAMMER motif is highly conserved in species ranging from yeast to humans and including rice (Fig. 1B). A phylogenic analysis of the LAMMER proteins from different organisms indicated that they form four separate groups (Fig. 1C). All the proteins from mammals and insects are in group I. The proteins from both monocots and dicots fall into group II. OsDR11L is the most closely related to AtAFC2, one of the LAMMER kinases in Arabidopsis (Fig. 1C). Fungal LAMMER proteins are in group III, and group IV only includes LAMMER protein from the malaria parasite *Plasmodium falciparum* (Fig. 1C).

The results collectively suggest that *OsDR11* has at least two alternative transcripts, *OsDR11S* and *OsDR11L*, in rice variety Minghui 63. They also suggest that *OsDR11L* encodes a LAMMER kinase-type protein.

Analysis of the Relationship of *OsDR11* and Quantitative Resistance

Minghui 63 is resistant to *Xoo* by both qualitative resistance, conferred by *MR* genes *Xa3/Xa26* and *xa25*, and quantitative resistance, conferred by resistance QTLs (Sun et al., 2004; Xiang et al., 2006; Hu et al., 2008; Liu et al., 2011). A recombinant inbred line segregation population developed from a cross between susceptible Zhenshan 97 and Minghui 63 has been used to study quantitative disease resistance against *Xoo*, and several defense-responsive genes contributing to resistance QTLs have been characterized by using this population (Qiu et al., 2007; Ding et al., 2008; Hu et al., 2008; Kou

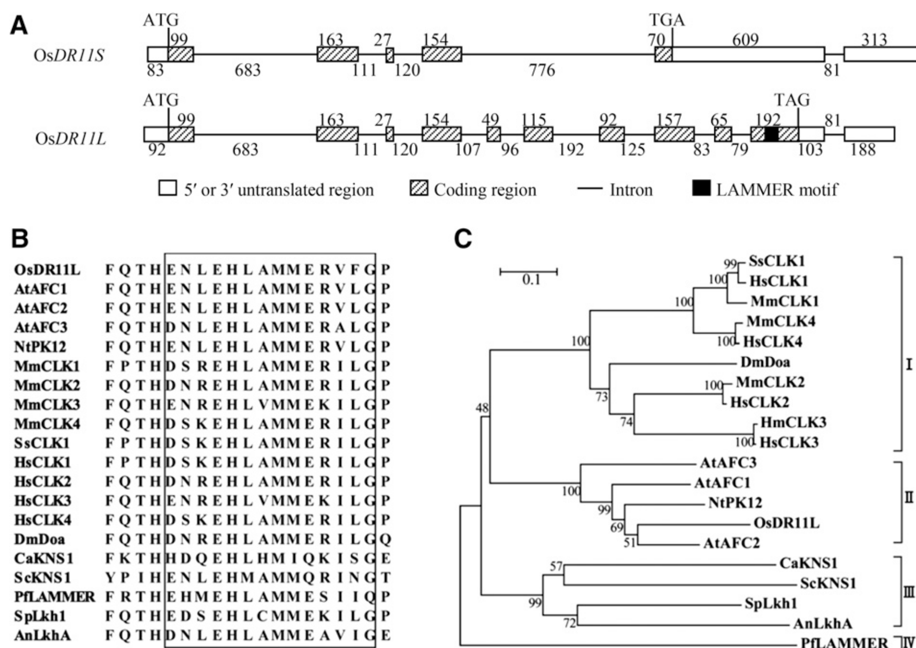

Figure 1. Analysis of *OsDR11* and encoded product sequences. A, Structures of *OsDR11S* and *OsDR11L*. ATG, Translation start codon; TGA or TAG, translation stop codon. The numbers indicate the nt of each substructure. B, Alignment of multiple amino acid sequences of LAMMER motifs (framed) from different species. Protein accessions: Arabidopsis AtAFC1 (P51566), AtAFC2 (P51567), and AtAFC3 (P51568); *N. tabacum* NtPK12 (AAC04324); *Mus musculus* MmCLK1 (NP_001036099), MmCLK2 (NP_001156904), MmCLK3 (NP_031739), and MmCLK4 (NP_031740); *Sus scrofa* SsCLK1 (EU431337); *Homo sapiens* HsCLK1 (NP_001155879), HsCLK2 (NP_003984), HsCLK3 (NP_001123500), and HsCLK4 (NP_065717); *Drosophila melanogaster* DmDOA (P49762); *Plasmodium falciparum* PfLammer (AAK38173); *Candida albicans* CaKNS1 (XP_722185); *Saccharomyces cerevisiae* ScKNS1 (P32350); *Schizosaccharomyces pombe* SpLkh1 (Q10156); *Aspergillus nidulans* AnLkhA (XP_658592). C, Phylogenetic relationship of LAMMER family proteins in different species.

et al., 2010; Deng et al., 2012). We also used this population to comap *OsDR11* and resistance QTLs. The mapping showed that *OsDR11* was colocalized with the peak of a minor resistance QTL, which had a logarithm of odds of 2.51, on rice chromosome 12 (Fig. 2A; Chen, 2001). This resistance QTL explained 4.82% of the phenotypic variation against *Xoo* and was contributed by an allele from resistance Minghui 63 in this population.

To ascertain the tissue-specific expression characteristics of *OsDR11* in Minghui 63, we examined the two transcripts in different tissues collected throughout its life cycle. In general, both *OsDR11S* and *OsDR11R* were continuously expressed throughout the life cycle, but they showed different expression patterns (Fig. 2B). *OsDR11S* expression was higher in callus, root, leaf, sheath, and pistil, while *OsDR11L* expression was more abundant in shoot, panicle, and stamen (Fig. 2B).

Since the leaf tissue is the major site of *Xoo* invasion (Kou and Wang, 2013), we next checked the expression of *OsDR11S* and *OsDR11L* in leaves of two pairs of rice lines after *Xoo* infection. The first pair of rice lines was Minghui 63 and Zhenshan 97. Minghui 63 has resistance to some *Xoo* strains, which is conferred by *MR* gene *Xa3/Xa26*, but Zhenshan 97 is highly susceptible to *Xoo* (Sun et al., 2004; Xiang et al., 2006). The second pair of rice lines was transgenic line Rb49 and japonica rice variety Mudanjiang 8. Rb49, with the genetic background of Mudanjiang 8, carries *Xa3/Xa26* and is highly resistant to some *Xoo* strains, and Mudanjiang 8 is highly susceptible to *Xoo* (Cao et al., 2007). In susceptible Zhenshan 97, both *OsDR11S* and *OsDR11L* had similar expression patterns; they were slightly induced at 4 to 8 h after inoculation, returned to normal level at 48 h, and then reached their peak level at 84 h (Fig. 2C). In contrast, the expression patterns of the two variants in resistant Minghui 63 were totally different. There was an early (8 h) and a late (84 h) increase of *OsDR11S* expression level after a slight suppression, but no obvious induction peak occurred in *OsDR11L* expression after *Xoo* infection (Fig. 2C). The same results were observed in another pair of rice lines, Mudanjiang 8 and Rb49. The two variants had a similar expression pattern in susceptible Mudanjiang 8 after *Xoo* infection, with expression being slowly induced and reaching a peak at 12 to 24 h (Fig. 2C). In contrast, the two variants had distinct expression patterns in the resistant Rb49. *OsDR11S* had relatively stable expression in both Mudanjiang 8 and Rb49, but the expression level was significantly higher in the resistant line than in the susceptible line, either with or without pathogen infection. *OsDR11L* was rapidly suppressed after infection in

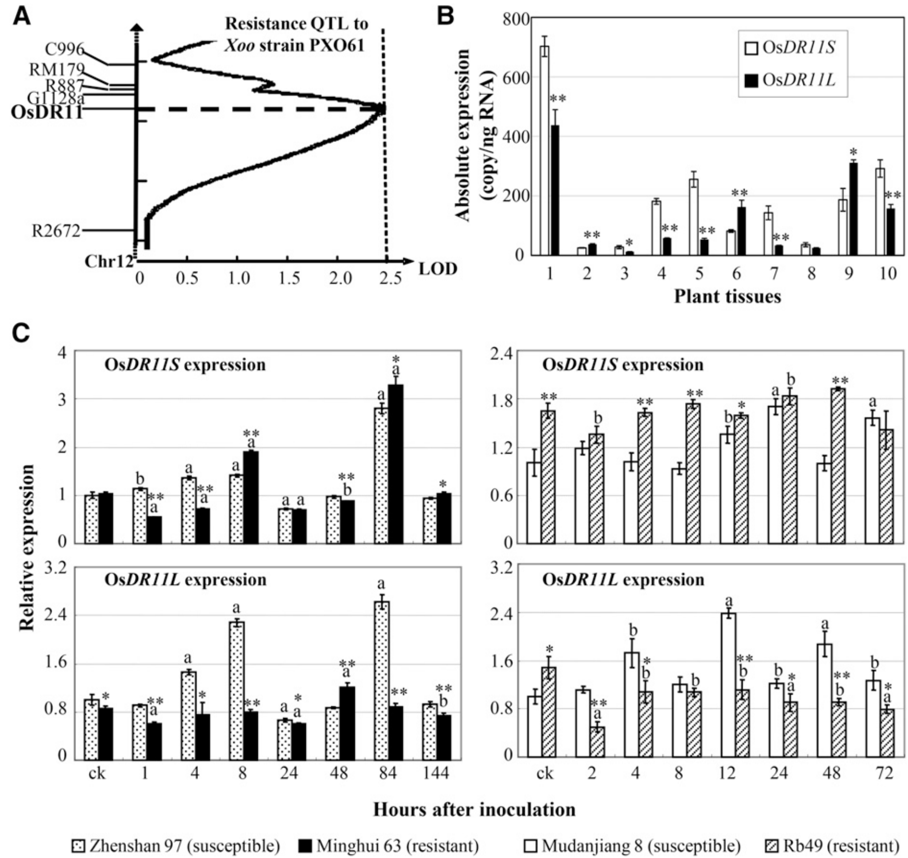

Figure 2. The features of *OsDR11*. A, Colocalization of *OsDR11* with a minor disease resistance QTL against *Xoo*. B, Expression of *OsDR11S* and *OsDR11L* during development in rice variety Minghui 63. 1, Callus; 2, shoot at the two-tiller stage; 3, root at the two-tiller stage; 4, leaf at the booting (panicle development) stage; 5, sheath at the booting stage; 6, panicle at the booting stage; 7, flag leaf at the heading stage; 8, panicle at the heading stage; 9, stamen at the flowering stage; 10, pistil at the flowering stage. Asterisks indicate significant difference between two rice lines with the same treatment at $^{**}P < 0.01$ or $^{*}P < 0.05$. C, *OsDR11S* and *OsDR11L* expression after pathogen infection. Plants were inoculated with *Xoo* strain PXO61 at the booting stage. The letter "a" or "b" above the bars indicates significant difference between pathogen-infected and noninfected (ck) plants in the same rice line at $P < 0.01$ or $P < 0.05$, respectively.

Rb49, and the level remained significantly lower in the resistant line compared with Mudanjiang 8 after *Xoo* infection (Fig. 2C).

In summary, quantitative resistance result suggests that *OsDR11* may contribute to a minor resistance QTL against *Xoo* and the distinct expression patterns of *OsDR11S* and *OsDR11L* in resistant rice lines after pathogen inoculation suggest that *OsDR11S* and *OsDR11L* may be differentially involved in rice-*Xoo* interaction.

Analysis of the Roles of the Two *OsDR11* Variants in Rice-*Xoo* Interaction

To investigate whether *OsDR11S* and *OsDR11L* were involved differently in rice defense to *Xoo* infection, we manipulated the expression of both *OsDR11S* and *OsDR11L*. Fifteen independent positive *OsDR11S*-overexpressing (oe) plants (also named D35UM8) were obtained. Seven of the 15 T_0 *OsDR11S*-oe plants were significantly more resistant ($P < 0.05$) to *Xoo*, with lesion areas ranging from 14% to 39%, compared to 60% for wild-type Mudanjiang 8 (Supplemental Table S1). To confirm that the enhanced resistance of the transgenic plants was due to overexpression of *OsDR11S*, two T_1 families were further analyzed individually for *Xoo* resistance and their *OsDR11S* expression level. All T_1 plants showing significantly enhanced ($P < 0.01$) resistance had increased expression of *OsDR11S*, and wild-type siblings had no significant differences from wild-type plants (Fig. 3A). While the transgenic lines showed increased resistance, it was not directly correlated with the expression levels. Such as the expression levels of plants 18, 22, and 25 did not correlate with resistance levels in the *OsDR11S*-oe33 T_1 family (Fig. 3A), which may suggest that above a certain level of expression of *OsDR11S* will make no difference. We also generated *OsDR11S-Flag*-oe plants (D230UM). The phenotypes of these plants were the same as the *OsDR11S*-oe plants, which did not carry the Flag tag. The reduced lesion area of *OsDR11S-Flag*-oe plants caused by *Xoo* significantly negatively correlated with increased expression of *OsDR11S* ($r = -0.606$, $n = 15$, $P < 0.05$ in *OsDR11S-Flag*-oe2 family; $r = -0.661$, $n = 14$, $P < 0.05$ in *OsDR11S-Flag*-oe7 family; Supplemental Fig. S2).

Fifteen independent positive *OsDR11S*-suppressing (D205RM) plants were obtained by using an artificial microRNA (ami) strategy. These *OsDR11S*-ami plants showed a phenotype that was opposite to that of *OsDR11S*-oe plants. Twelve of the 15 positive T_0 plants showed significantly increased susceptibility ($P < 0.05$) to *Xoo*, with lesion areas ranging from 62.6% to 78.3%, compared to 56.7% for wild-type Mudanjiang 8 (Supplemental Table S2). Two T_1 families were further analyzed for their *Xoo* resistance and *OsDR11S*

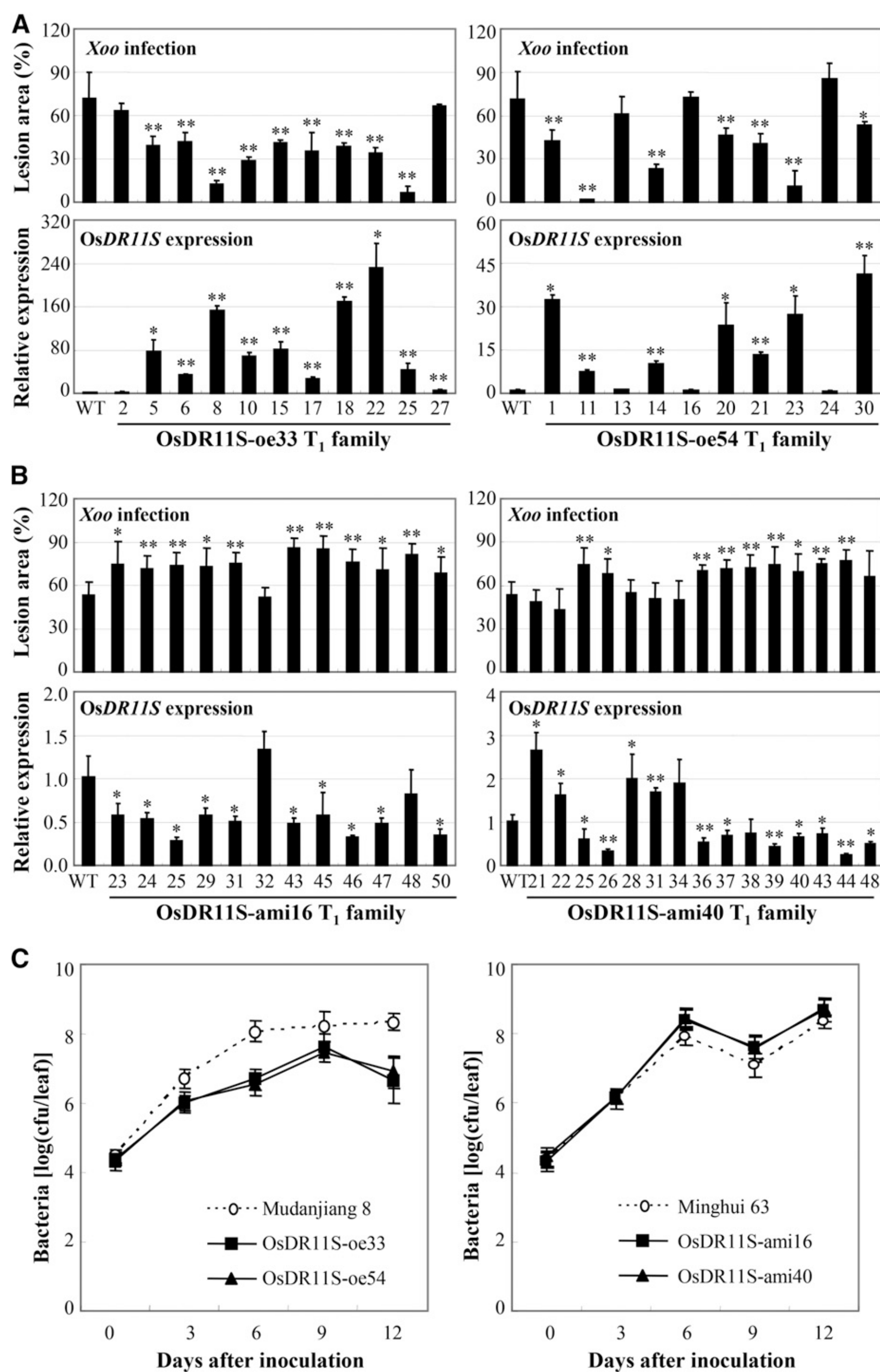

Figure 3. Modulating *OsDR11S* expression influenced the resistance to *Xoo*. Rice plants were inoculated with *Xoo* strain PXO99 at the booting stage. Data represent mean (three to six replicates from one plant for disease area, three replicates from one plant for gene expression, and three replicates from each type of plant for bacterial growth) ± SD. Asterisks indicate significant difference between transgenic and wild-type (WT) plants at $^{**}P < 0.01$ or $^{*}P < 0.05$. A, Enhanced resistance to *Xoo* was associated with overexpression of *OsDR11S* in T1 families. WT, Mudanjiang 8. B, Increased susceptibility to *Xoo* was associated with suppression of *OsDR11S* in T1 families. WT, Minghui 63. C, Modulating *OsDR11S* expression influenced the growth of PXO99 in rice leaves. 0 h, Immediately after inoculation; cfu, colony-forming unit.

expression level. The increased susceptibility of T_1 plants significantly negatively correlated with reduced *OsDR11S* transcripts ($r = -0.651$, $n = 13$, $P < 0.05$ in *OsDR11S*-ami16 family; $r = -0.852$, $n = 16$, $P < 0.001$ in *OsDR11S*-ami40 T1 family; Fig. 3B).

Bacterial growth results reflected the phenotype of the transgenic plants (Fig. 3C). The growth rates of bacteria on the leaves of *OsDR11S*-oe plants were 7.4- to 20.3-fold lower than on the susceptible wild-type plant leaves at 6 to 12 d after infection. In *OsDR11S*-ami plants, the rates were 3.7- to 7.7-fold higher than in the wild-type plant at 6 to 12 d after infection (Fig. 3C). These results suggest that *OsDR11S* may act as a positive regulator in the rice response to *Xoo* infection.

Nineteen independent positive *OsDR11L*-oe (D126UM) plants and eight independent positive *OsDR11L-Flag*-oe (D231UM) plants were generated. However, overexpressing *OsDR11L* appears having no obvious effect on rice response to *Xoo* infection (Supplemental Tables S3 and S4; Supplemental Fig. S3).

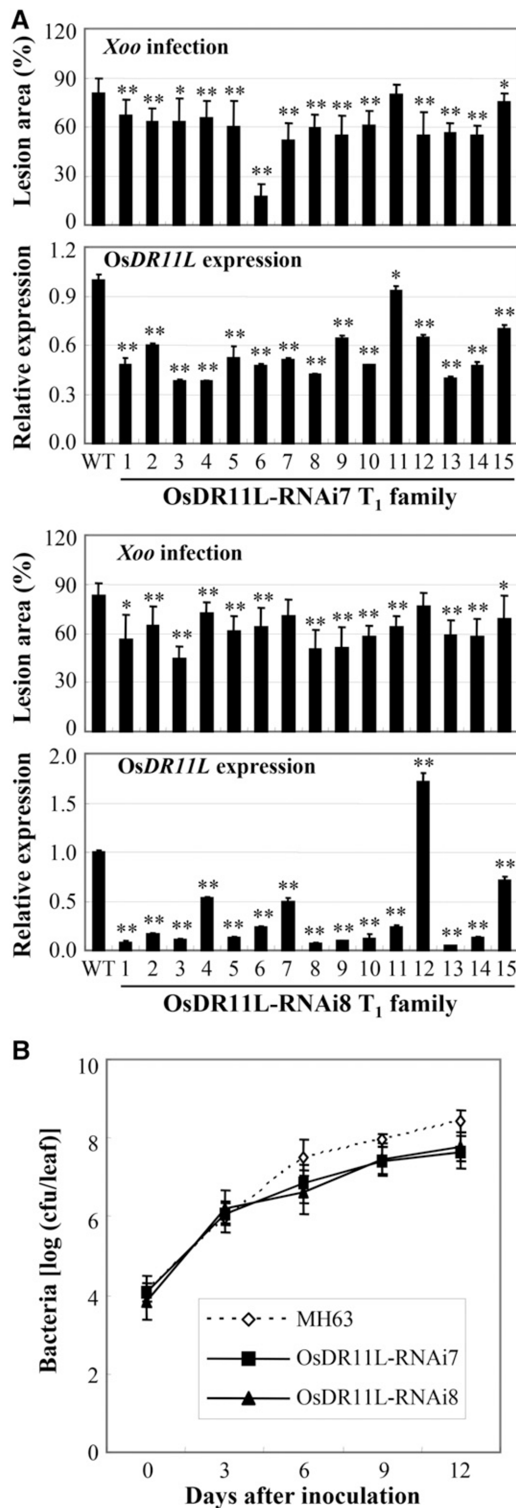

Figure 4. Suppressing *OsDR11L* expression enhanced the resistance to *Xoo*. Rice plants were inoculated with *Xoo* strain PXO99 at the booting stage. Data represent mean (three to six replicates from one plant for disease area, three replicates from one plant for gene expression, and three replicates from each type of plant for bacterial growth) ± SD. Asterisks indicate significant difference between transgenic and wild-type (WT) plants at $^{**}P < 0.01$ or $^{*}P < 0.05$. A, Enhanced resistance to *Xoo* cosegregated with suppression of *OsDR11L* in T1 families. B, Suppressing *OsDR11L* expression reduced the growth of *Xoo* in rice leaves. 0 h, Immediately after inoculation; cfu, colony-forming unit.

Ten independent positive *OsDR11L*-suppressing plants (D229RM) were generated in rice variety Minghui 63 by using the RNA interference (RNAi) strategy. Seven of the 10 T_0 *OsDR11L*-RNAi plants were significantly less susceptible to *Xoo* ($P < 0.05$), with lesion areas ranging from 64.3% to 74.3%, compared to 97.2% for wild-type Minghui 63 (Supplemental Table S5). Two T_1 families were further analyzed for their *Xoo* resistance and *OsDR11L* expression level. The reduced susceptibility of T_1 plants significantly correlated with reduced *OsDR11L* transcription ($r = 0.528$, $n = 16$, $P < 0.05$ in *OsDR11L*-RNAi7 family and $r = 0.773$, $n = 16$, $P < 0.01$ in *OsDR11L*-RNAi8 family; Fig. 4A). The bacteria growth rate in *OsDR11L*-RNAi plants was 6.0- to 11.9-fold lower than in wild-type Minghui 63 at 6 to 12 d after infection (Fig. 4B). These results suggest that *OsDR11L* may act as a negative regulator in the rice response to *Xoo* infection.

Analysis of *OsDR11*-Related Defense Pathway in Rice-*Xoo* Interaction

Salicylic acid (SA)-dependent signaling and JA-dependent signaling are important pathways in plant resistance to pathogens. To identify a signaling pathway in which *OsDR11S* and *OsDR11L* were possibly involved, we quantified the concentrations of the endogenous phytohormones SA, JA, and JA-Ile, which is an active metabolite of JA and involved in biotic challenges (Liu et al., 2012), in rice leaves. The SA level was slightly lower in *OsDR11S*-oe plants compared to wild-type plants, and it generally showed no obvious difference in *OsDR11S*-ami and *OsDR11L*-RNAi plants compared to their corresponding wild-type plants (Fig. 5). Before or after *Xoo* infection, the JA and JA-Ile levels were both significantly higher ($P < 0.05$) in *OsDR11S*-oe plants and *OsDR11L*-RNAi plants than in wild-type Mudanjiang 8 and Minghui 63, respectively. In contrast, the JA and JA-Ile levels were significantly lower ($P < 0.05$) in *OsDR11S*-ami plants than in wild-type Minghui 63 at most of the time points examined (Fig. 5).

Consistent with the altered concentration of JA and JA-Ile in different transgenic plants, the expression of a set of pathogen-induced defense-responsive genes was also changed in these transgenic plants. *Lipoxygenase* (D14000) and *allene oxide synthase2* (AY062258) are involved in JA synthesis (Lyons et al., 2013), and the expression of *basic pathogenesis-related protein1* (U89895) and *phytoalexin-deficient4* (AK243523) have been reported to be influenced by both SA and JA signaling (Qiu et al., 2007; Tao et al., 2009; Shen et al., 2011; Ke et al., 2014). *Xoo* infection influenced the expression of all four genes (Fig. 6). The expression of *lipoxygenase*, *allene oxide synthase2*, and *basic pathogenesis-related protein1* in *OsDR11S*-oe and *OsDR11L*-RNAi plants was significantly

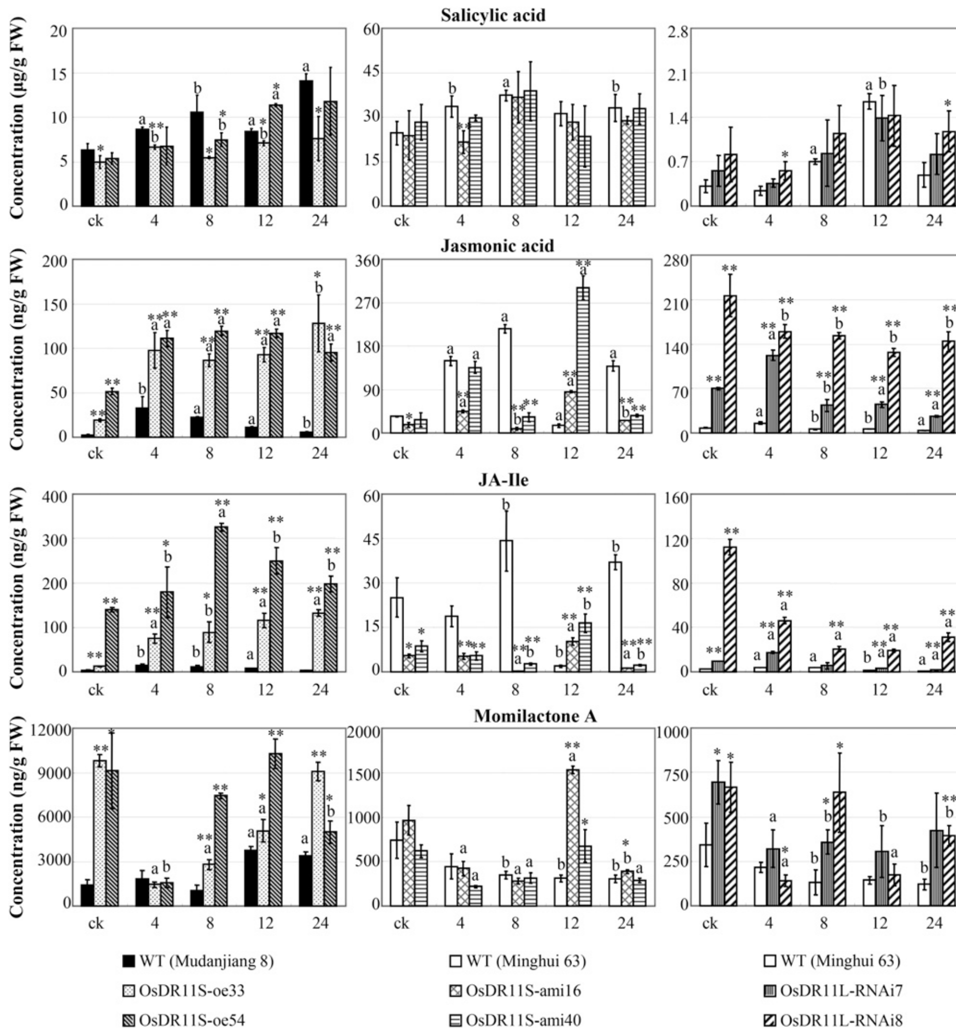

Figure 5. Modulating *OsDR11S* and *OsDR11L* expression influenced the levels of phytohormones and phytoalexin. Rice plants were inoculated with *Xoo* strain PXO99 at the booting stage. Each data point represents ten replicates of leaf samples from ten different individual plants in one line. Bars represent mean (three replicates) ± SD. The letter "a" or "b" above the bars indicates significant difference between pathogen-infected and non-infected (ck) plants in the same rice line at $P < 0.01$ or $P < 0.05$, respectively. Asterisks indicate significant difference between transgenic and wild-type (WT) plants at $^{**}P < 0.01$ or $^{*}P < 0.05$.

increased compared to their corresponding wild-type plants both before and after *Xoo* infection. In contrast, the expression levels of the three genes were significantly lower or not significantly different in *OsDR11S*-ami plants compared to wild-type plants at most of the time points examined. The expression level of *phytoalexin-deficient4* was slightly higher in *OsDR11S*-oe and *OsDR11L*-RNAi plants compared to wild-type plants, but it generally showed no obvious difference between *OsDR11S*-ami and wild-type plants.

Phytoalexins serve as antibiotics in response to biotic stresses. The terpenoid momilactone A is an important phytoalexin in rice (Liu et al., 2012). The momilactone A was significantly higher in *OsDR11S*-oe and *OsDR11L*-RNAi plants both before and after *Xoo* infection, but its level in *OsDR11S*-ami plants did not differ from the wild-type control at most of the time points examined (Fig. 5).

These results suggest that *OsDR11S*-promoted disease resistance may be associated with activation of the JA-dependent pathway and accumulation of phytoalexin momilactone A, while *OsDR11L*-suppressed disease resistance may be associated with suppression of both JA-dependent signaling and accumulation of momilactone A.

Analysis of the Biochemical Features of the Two Alternatively Spliced Forms of *OsDR11*

To investigate the possible biochemical function of OsDR11S and OsDR11L proteins, their subcellular localizations were determined by fusing their respective coding regions in frame to the yellow fluorescence protein (YFP) reporter gene under the control of the 35S promoter. The fusion genes were transiently expressed in rice protoplast cells (Fig. 7). Similar to other members of the LAMMER family in other species, the OsDR11 proteins contain a putative nuclear localization signal at the amino terminus. So, we cotransformed the target gene with *Ghd7*-cyan fluorescence protein (CFP) gene. Rice Ghd7 has been used as a marker since it was reported as a transcription factor localized in the nucleus (Xue et al., 2008). The results showed that the OsDR11S-YFP and OsDR11L-YFP fusion proteins, respectively, colocalized with the marker in nucleus, suggesting that OsDR11S and OsDR11L may function in the nucleus.

To determine whether *OsDR11S* and *OsDR11L* encode functional protein kinases, the coding regions of *OsDR11S* and *OsDR11L* were fused in frame to the maltose-binding

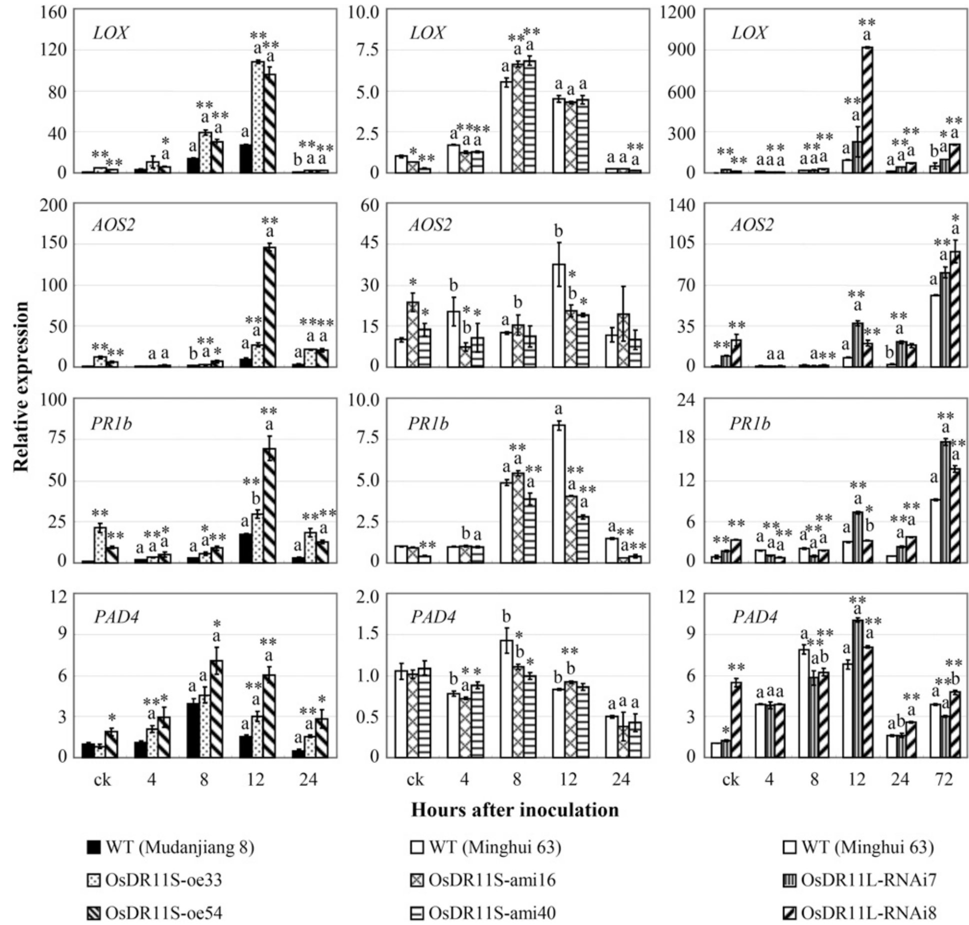

Figure 6. Modulating the expression of *OsDR11S* and *OsDR11L* influenced the expression of other defense-responsive genes analyzed by quantitative reverse transcription PCR. Rice plants were inoculated with *Xoo* strain PXO99 at the booting stage. Each data point represents ten replicates of leaf samples from ten different individual plants in one line. Bars represent mean (three replicates) ± SD. The letter "a" or "b" above the bars indicates significant difference between pathogen-infected and noninfected (ck) plants in the same rice line at $P < 0.01$ or $P < 0.05$, respectively. Asterisks indicate significant difference between transgenic and wild-type (WT) plants at $^{**}P < 0.01$ or $^{*}P < 0.05$.

protein (MBP) and expressed and purified in bacteria (Fig. 8A). The OsDR11S-MBP and OsDR11L-MBP recombination proteins had the expected molecular masses of 60 kD and 80 kD (Fig. 8A). The in vitro kinase assay showed that the OsDR11L-MBP had autophosphorylation activity, while OsDR11S-MBP and MBP did not (Fig. 8B). Both OsDR11S-MBP and OsDR11L-MBP were unable to phosphorylate the myelin basic protein as substrate (Fig. 8C). OsDR11L could not phosphorylate OsDR11S as substrate, but an increased OsDR11S concentration resulted in decreased autophosphorylation of OsDR11L (Fig. 8C). Next, we used western blot analysis to confirm that the inactivation of OsDR11L kinase activity in the presence of OsDR11S was not due to protein degradation (Fig. 8D). These results suggested that OsDR11S can inactivate or inhibit the kinase activity of OsDR11L.

Analysis of the Influence of *OsDR11S* on the Transcriptional Expression of *OsDR11L*

To study whether *OsDR11S* influenced *OsDR11L* expression in vivo, we further analyzed the expression level of *OsDR11L* in *OsDR11S*-oe plants. Two *OsDR11S*-oe T1 families were analyzed individually for *OsDR11L* expression. Most of T_1 plants overexpressing *OsDR11S* showed significantly suppressed expression ($P < 0.05$) of *OsDR11L* compared to wild-type plants (Fig. 9A). We further analyzed the expression levels of *OsDR11S* and *OsDR11L* in F_1 plants that were generated from a cross between the *OsDR11S-Flag*-oe plants

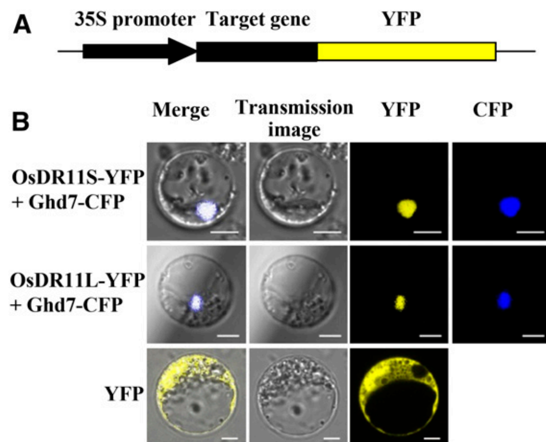

Figure 7. Subcellular localization of OsDR11. Ghd7-CFP, nuclear localization marker. A, The schematic diagram indicates the construct used for protein location. B, OsDR11S and OsDR11L located in rice nuclear.

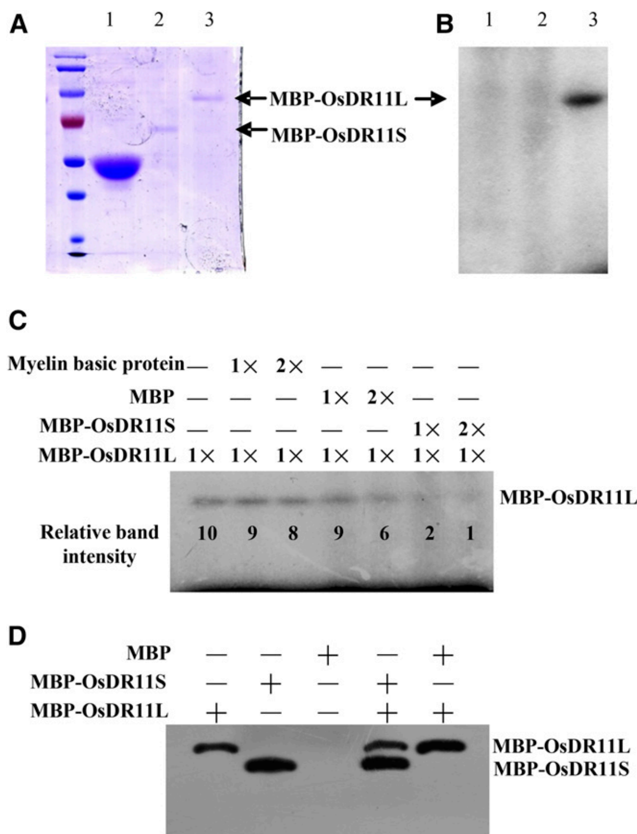

Figure 8. The ability of OsDR11L to undergo autophosphorylation was influenced by the presence of OsDR11S. A, Purification of proteins. 1, MBP; 2, recombination protein MBP-OsDR11S; 3, recombination protein MBP-OsDR11L. B, Autophosphorylation assay in vitro. 1, MBP; 2, MBP-OsDR11S; 3, MBP-OsDR11L. C, The ability of autophosphorylation of OsDR11L was influenced by the presence of OsDR11S in vitro. The numbers under each band are the relative ratios of the OsDR11L kinase signals. D, The proteins detected by western blotting using anti-OsDR11 antibody.

and *OsDR11L-Flag*-oe plants. The expression levels of *OsDR11L* in F1 plants that had high expression levels of *OsDR11S* were lower compared to the levels of *OsDR11L* in their parent *OsDR11L*-oe line, and the expression levels of *OsDR11S* and *OsDR11L* were significantly negatively correlated in these plants ($r = -0.545$, $n = 14$, $P < 0.05$) (Fig. 9B). The expression levels of *OsDR11L* in six positive F_1 plants (3 to 6, 9, and 11) were only 7% to 23% of those in *OsDR11L-Flag*-oe plants, while the expression levels of *OsDR11S* in these positive F_1 plants were 49% to 89% of those in *OsDR11S-Flag*-oe plants. Consistent with the expression features of *OsDR11S* and *OsDR11L*, the F_1 and F_2 plants overexpressing both *OsDR11S* and *OsDR11L* had lesion areas similar to those of the *OsDR11S-Flag*-oe line after *Xoo* infection (Fig. 9B; Supplemental Fig. S4). The expression levels of *OsDR11S* and *OsDR11L* significantly associated with lesion area caused by *Xoo* ($r^2 = 0.717$, $P = 0.001$).

The coding sequences of *OsDR11S* and *OsDR11L* were further analyzed. The first 443 nt of *OsDR11S* were identical to those of *OsDR11L*, and the last 70 nt of *OsDR11S* were identical to nt 700–769 of *OsDR11L*. There seemed to be a dynamic balance between the expression of *OsDR11S* and *OsDR11L*. When expression of *OsDR11S* was high, the transcription of *OsDR11L* may have been reduced by a negative regulation mechanism. These results suggest that *OsDR11S* can influence *OsDR11L* transcript abundance in vivo.

DISCUSSION

Although LAMMER proteins have been well studied in some species, such as in mice, humans, Arabidopsis, and tobacco, and they are reported to have functions in differentiation and development (Nikolakaki et al., 2002; Talevich et al., 2011; Yun et al., 1994), the exact role of LAMMER kinase in rice is poorly understood. Here, we provide evidence that LAMMER protein kinase gene *OsDR11* is involved in rice-*Xoo* interaction.

Two Variants of *OsDR11* Play Opposite Roles in Rice-*Xoo* Interaction

Alternative splicing of mRNAs is an important biological phenomenon of gene transcription, although how the alternatively spliced transcripts function differently in a given genome is largely unknown. Alternative splicing of LAMMER kinase gene has also been reported in Arabidopsis, but the functions of the premature termination codon transcripts generated through alternative splicing are unknown (Marquez et al., 2012). LAMMER kinases appear to play vital roles in various physiological processes, but their roles in plant defense are not yet known. The present results suggest that the two alternative spliced transcripts of *OsDR11* are involved in rice-*Xoo* interaction but function differently.

Many protein kinases function as either positive or negative components in disease resistance against a pathogen species (Matsui et al., 2010; Christiansen et al., 2011; Shen et al., 2011). However, a protein kinase serving as both a positive and a negative regulator in defense responses to the same pathogen species has been reported (Shen et al., 2010). The present results on *OsDR11S* and *OsDR11L* in the context of rice-*Xoo* interaction provide another example that a protein kinase gene can play opposite roles via alternatively spliced transcripts. This hypothesis is supported by the following evidence. First, the *OsDR11S* expression level was significantly higher in the resistant line than in the susceptible line after *Xoo* infection, but *OsDR11L* was more abundant in the susceptible line after *Xoo* infection (Fig. 2C). Second, overexpressing *OsDR11S* and suppressing *OsDR11L* increased rice resistance to *Xoo*, and suppressing *OsDR11S* increased susceptibility (Figs. 3 and 4). These results suggest that both variants of *OsDR11* have roles in rice-*Xoo* interaction, with

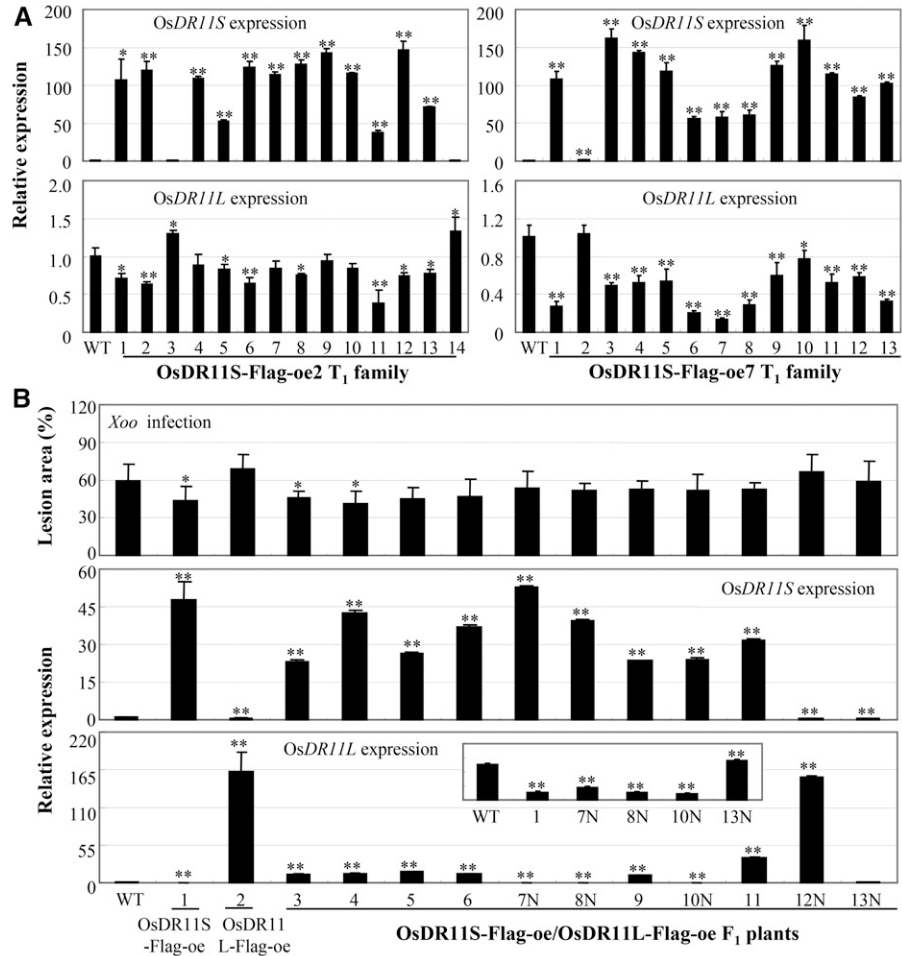

Figure 9. Overexpressing *OsDR11S* suppressed the expression of *OsDR11L*. WT, Mudanjiang 8. A, The expression level of *OsDR11L* in *OsDR11S-Flag*-oe T_1 plants. B, The phenotype to *Xoo* and expression of *OsDR11L* and *OsDR11S* of the F_1 plants generated from the cross between *OsDR11S-Flag*-oe and *OsDR11L-Flag*-oe lines. 7N, 8N, 10N, 12N, and ^{13}N, Negative F_1 plants. Asterisks indicate significant difference between transgenic and wild-type plants at $^{**}P < 0.01$ or $^{*}P < 0.05$.

OsDR11S functioning as a positive regulator, while *OsDR11L* functions as a negative one.

OsDR11-Mediated Disease Resistance May Be Associated with Activation of the JA-Dependent Pathway and Accumulation of Phytoalexin Momilactone A

SA and JA are important phytohormone molecules in plant-pathogen interactions. SA is generally involved in the activation of defense responses against biotrophic and hemibiotrophic pathogens, and JA is usually associated with defense against necrotrophic pathogens (Bari and Jones, 2009). *Xoo* is a biotrophic pathogen (Li and Wang, 2013). For example, the enhanced *Xoo* resistance of *OsWRKY13*-oe rice plants and *OsDR10*-suppressing rice plants was associated with activation of the SA-dependent pathway and suppression of the JA-dependent pathway (Qiu et al., 2007, 2008; Xiao et al., 2009). However, the present results suggest that *OsDR11*-mediated rice resistance to *Xoo* is accompanied by activation of the JA-associated pathway, but not the SA pathway. This inference is supported by the following evidence. First, the enhanced resistance of *OsDR11S*-oe plants and *OsDR11L*-suppressing plants was associated with increased endogenous levels of JA and its active metabolite JA-Ile, but not SA (Fig. 5). Second, although *Xoo* is a biotrophic pathogen, rice resistance against *Xoo* appears to employ complex regulatory mechanisms to trigger effective defense responses in natural environments. For example, the enhanced *Xoo* resistance in *OsMPK6*-oe rice plants and *OsEDR1*-knockout rice plants was associated with activation of both SA- and JA-associated pathways (Shen et al., 2010, 2011). The *Xoo* resistance mediated by rice *OsWRKY45-2* or *C3H12* was associated with JA, but not SA (Tao et al., 2009; Deng et al., 2012). Furthermore, exogenous application of JA can enhance the resistance of rice to *Xoo* (Yamada et al., 2012; Ke et al., 2014).

Phytoalexins contribute to plant basal immunity and accumulate around infection sites soon after pathogen attack (González-Lamothe et al., 2009). Our results also show that the endogenous accumulation of momilactone A was significantly higher in *OsDR11S*-oe plants and *OsDR11L*-suppressing plants before and after *Xoo* infection, which was associated with resistance against *Xoo* infection and accumulation of JA (Fig. 5). Previous studies showed that the accumulation level of momilactone A can be induced by infection of rice pathogens such as *Xoo* and fungal *Magnaporthe oryzae*

(Liu et al., 2012; Riemann et al., 2013). Momilactone A can inhibit the growth of *M. oryzae*, and a reduced level of momilactone A is associated with compromised resistance against *Xoo* (Hasegawa et al., 2010; Ke et al., 2014). Exogenous treatment with JA increased accumulation of momilactone A (Nojiri et al., 1996; Kato-Noguchi, 2011). Thus, this study suggests that *OsDR11S*-mediated rice resistance may also be associated with the accumulation of momilactone A, which may be positively regulated by JA signaling.

OsDR11S May Regulate the Function of OsDR11L

The LAMMER motif of LAMMER kinases is important for kinase activity (Savaldi-Goldstein et al., 2000). This may explain why OsDR11L showed autophosphorylation ability while OsDR11S, which lacks LAMMER motif, did not (Fig. 8). Although OsDR11S does not have autophosphorylation ability, our results suggest that OsDR11S appears to be a dominant and functional variant. This inference is supported by evidence that the F_1 and F_2 plants overexpressing both *OsDR11S* and *OsDR11L* showed the phenotype of *OsDR11S*-oe plants in response to *Xoo* infection.

OsDR11L negatively influenced rice resistance to *Xoo*. The fine balance of *OsDR11S* with respect to *OsDR11L* is an important feature in *OsDR11* gene regulation. This hypothesis is supported by the following evidence. First, alternative splicing plays a role in the functional complexity of rice, and 59% of the alternative splicing events in rice were organ specific (Lu et al., 2010). In our results, both alternatively spliced variants are continuously expressed throughout the rice life cycle (Fig. 2B) and the expression level of both variants is influenced by *Xoo* infection (Fig. 2C), which suggest that alternative splicing of *OsDR11* is needed throughout rice development and in response to pathogen infection. Second, because of the pretermination codon by alternative splicing, *OsDR11S* likely regulates the expression level of *OsDR11L* by influencing the alternative splicing of *OsDR11*. Increasing evidence shows that alternative splicing coupled to mRNA degradation not only removes aberrant mRNAs but also acts as a mechanism to regulate gene expression, including the expression of genes playing essential roles in plant stress adaptation (Dubrovina et al., 2013). In this study, the expression of *OsDR11L* was reduced in *OsDR11S*-oe plants and in F_1 plants generated from the cross between *OsDR11S*-oe and *OsDR11L*-oe plants (Fig. 9B), which suggests that overexpression of *OsDR11S* can regulate the alternative splicing of *OsDR11*. These results were consistent with LAMMER kinase studies in other species. For example, overexpressing *Clk/Sty* in mammalian cells leads to a switch in the alternative splice site selection of miniature gene constructs (Duncan et al., 1997), and PK12 LAMMER kinase modulates alternative splicing in plants in a dose-dependent manner (Savaldi-Goldstein et al., 2003). However, the mechanism of how overexpressing *OsDR11S* can reduce expression level of *OsDR11L* needs further studies.

OsDR11S may also regulate OsDR11L function by influencing its kinase activity. This inference can be supported by the fact that autophosphorylation ability of OsDR11L was inhibited by the presence of OsDR11S, not the degradation of OsDR11L (Fig. 8). Both OsDR11S and OsDR11L were located in the nucleus. This colocalization provides the foundation for OsDR11S being able to affect OsDR11L. However, physical interaction of the two types of proteins was not detected by either coimmunoprecipitation assay in vivo or biomolecular fluorescence complementation assay in rice protoplasts. Thus, further study is needed to elucidate how OsDR11S direct influences the kinase function of OsDR11L.

CONCLUSION

Rice LAMMER kinase gene *OsDR11* is alternatively spliced in rice-*Xoo* interaction. The two transcripts of *OsDR11*, *OsDR11L*, and *OsDR11S*, play opposite roles, as negative and positive regulators, respectively, in rice resistance to *Xoo*, which is associated with the JA-dependent pathway. OsDR11S appears to suppress the negative function of OsDR11L in rice-*Xoo* interaction by both transcriptionally suppressing *OsDR11L* and inhibiting the kinase activity of OsDR11L. In addition, *OsDR11* may contribute to a minor resistance QTL against *Xoo* through the function of *OsDR11S*. Elucidating the molecular mechanisms of the two alternatively spliced transcripts provide important insights into the regulatory mechanisms of alternatively spliced transcripts in rice.

MATERIALS AND METHODS

Gene Isolation and Structural Analysis

To isolate the *OsDR11* gene, two cDNA fragments of *OsDR11*, EI39C8 and EI114F3, from rice (*Oryza sativa*) variety Minghui 63 (Zhang et al., 2005) were used to screen the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) using the BLAST program (Altschul et al., 1997). A series of PCR primers (Supplemental Table S6) were designed for sequencing the genomic DNA of *OsDR11* from Minghui 63. The analysis indicated that cDNA sequences EI39C8 and EI114F3 from Minghui 63 were highly homologous to gene locus LOC_Os12g27520 and harbored the entire coding sequence. These two variants also contained the 3′ untranslated region of *OsDR11*. The structures of *OsDR11* were determined by comparatively sequencing the genomic DNA and cDNA.

QTL Analysis and DNA Gel Blot Analysis

A rice recombinant inbred line population was used to analyze comapping of *OsDR11* and resistance QTLs. This population consisted of 241 lines developed from a cross between susceptible indica Zhenshan 97 (*O. sativa* ssp. *indica*) and resistant indica Minghui 63 (*O. sativa* ssp. *indica*) by single-seed descent. Minghui 63 carried *MR* genes *Xa3/Xa26* and *xa25* for *Xoo* resistance (Sun et al., 2004; Xiang et al., 2006; Liu et al., 2011). A molecular linkage map containing 221 markers and covering the whole rice genome was developed with this population (Xing et al., 2002). This population had been used to study the quantitative disease resistance for four strains (Chinese strains JL691 and KS-1-21 and Philippines strains PXO61 and PXO339) of *Xoo* (Chen, 2001; Hu et al., 2008). *OsDR11* was mapped into this population using cDNA EI39C8.

Mapmaker 3.0 was used for linkage analysis and QTL map for composite interval mapping at a threshold of logarithm of odds 2.5 (Li et al., 2001; Wang et al., 1999).

Rice total DNA was digested with restriction enzymes, separated by electrophoresis on 0.8% agarose gels, and blotted onto nylon membranes. DNA gel blot analysis was conducted as previously described, using the cDNA EI39C8 as probe for hybridization (Xiao et al., 2009).

Pathogen Inoculation

To evaluate bacterial blight disease, plants were inoculated with *Xoo* at the booting (panicle development) stage by the leaf-clipping method (Chen et al., 2002), and disease was scored by measuring the percentage of the lesion area (lesion length/leaf length) at 11 to 15 d after inoculation. *Xoo* growth rate in rice leaves was determined by counting colony-forming units (Sun et al., 2004).

Gene Expression Analyses

Two-centimeter leaf fragments next to bacterial infection sites were used for RNA isolation. Quantitative reverse transcription PCR was conducted as described previously (Qiu et al., 2007). To compare the expression levels of *OsDR11S* and *OsDR11L* during development, the known concentrations of plasmids EI39C8 and EI114F3 harboring *OsDR11S* and *OsDR11L*, respectively, were amplified as standards, and linear regression models were established to calculate the absolute expression of *OsDR11S* and *OsDR11L* in rice tissues. To analyze the relative expression of a gene, the expression level of the rice actin gene was used as an internal control, and the expression level relative to the control is presented. The relative expression level of a gene after treatment can be compared only to itself in corresponding control. To identify the two alternative forms of *OsDR11*, gene-specific primers 83J4-2F/83J4-2R were used to detect the expression of *OsDR11L* (Supplemental Table S6). For detecting the expression of *OsDR11S*, primers 39C8-5F/39C8-6R were used in wide-type, *OsDR11S*-oe, and *OsDR11S*-suppressing plants, and primers 39C8-6F/39C8-7R were used in *OsDR11S-Flag*-oe plants (Supplemental Table S6).

Transformation

The overexpression constructs of *OsDR11S* and *OsDR11L* were created by cutting the fragment harboring the full-length coding region from cDNA clones EI39C8 and EI114F3 by using restriction enzymes *Kpn*I and *Bam*HI, respectively, and ligating them into the transformation vector pU1301, which contained a maize ubiquitin gene promoter (Cao et al., 2007). To suppress *OsDR11S*, an artificial miRNA construct was generated according to a previous description (Warthmann et al., 2008). To construct an RNAi vector of *OsDR11L*, a 256-nt fragment amplified from Minghui 63 cDNA by using gene-specific primers (Supplemental Table S6) was inserted into the pDS1301 vector (Yuan et al., 2007). The constructs were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium*-mediated transformation was performed according to a published protocol (Lin and Zhang, 2005). The overexpressing construct was transferred into japonica rice variety Mudanjiang 8, and the suppressing constructs were transferred into indica rice variety Minghui 63. Primers for positive transplant test are listed in Supplemental Table S6.

Quantification of Phytohormones and Phytoalexin

Leaf fragments about 3 cm long next to the inoculation sites were used for analysis. Samples were prepared and quantified using the UFLC-ESI-MS/MS system as reported previously (Liu et al., 2012).

Subcellular Localization

To produce the constructs for assaying subcellular localization, the coding regions of *OsDR11S* and *OsDR11L* were amplified by PCR using gene-specific primers (Supplemental Table S6) and inserted into pM999-YFP for fusion with the reporter gene. Rice *Ghd7* has been used as a marker since it was reported as a transcription factor localized in the nucleus (Xue et al., 2008). The *35S:Ghd7-CFP* was kindly provided by Dr. Lei Wang of Huazhong Agricultural University. The constructs of OsDR11S-YFP or OsDR11L-YFP and Ghd7-CFP were cotransformed and transiently expressed in rice protoplasts according to a previous description (Yang et al., 2012).

Protein Expression and in Vitro Kinase Assay

The entire coding regions of *OsDR11S* and *OsDR11L* were amplified from cDNA clones EI39C8 and EI114F3, respectively, by PCR using gene-specific primers (Supplemental Table S6). After *Bam*HI and *Bam*HI/*Pst*I digestion, respectively, the PCR products of *OsDR11S* and *OsDR11L* were cloned into the pMAL-c2x vector, which harbors an MBP gene at the 5′ end of the multiple cloning sites (New England Biolabs). The expression plasmid was transformed into *Escherichia coli* strain BL21 (DE3). The MBP-tagged proteins were purified from *E. coli* using mylase resin (New England Biolabs).

For the autophosphorylation kinase activity assay, 1.2 μg of purified recombinant proteins, which were quantified using a Bradford assay (2-D Quant Kit; GE Healthcare), was incubated with kinase buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 0.3 μCi γ-^{32}P-ATP in a final volume of 20 μL. The mixture was incubated for 30 min at room temperature, and the reactions were terminated by the addition of protein sample buffer. Proteins were fractionated on a 12% SDS-PAGE gel and exposed to film. The kinase signals were calculated using Quantity one software (Bio-Rad, version 4.6).

Western blotting was performed as described previously (Yuan et al., 2009). The anti-OsDR11 antibody, which was custom synthesized by Abmart against peptide PRFASPPLRE (60th to 69th amino acid of both OsDR11L and OsDR11S), was used for protein expression analysis.

Statistical Analysis

The significant differences between control and treatment of the samples were analyzed by the pairwise *t* test in Excel (Microsoft). The correlation analysis between lesion area and gene expression level was performed using the CORREL analysis in Excel. R-squared value of the expression levels of *OsDR11S* and *OsDR11L* and lesion areas in *OsDR11S-Flag*-oe/*OsDR11L-Flag*-oe F_1 plants was analyzed using multiple linear regression analysis in SPSS (IBM SPSS Statistics, Version 19.0; IBM [released 2010]). A two-tailed $P < 0.05$ was considered statistically significant.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number KR107935 (*OsDR11*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. DNA gel blot analysis of *OsDR11* gene in rice varieties Minghui 63 (MH) and Zhenshan 97 (ZS).

Supplemental Figure S2. Enhanced resistance to *Xoo* was associated with overexpression of *OsDR11S* in two T_1 families.

Supplemental Figure S3. The response of *OsDR11L*-oe (A) and *OsDR11L-Flag*-oe (B) plants to infection by *Xoo* strain PXO99 was not closely associated with overexpression of *OsDR11L*, respectively.

Supplemental Figure S4. The F_2 plants overexpressing both *OsDR11S-Flag* and *OsDR11L-Flag* showed the phenotype of *OsDR11S-Flag*-oe line in response to *Xoo* infection.

Supplemental Table S1. The phenotype of *OsDR11S*-oe T_0 plants after inoculation with *Xoo* strain PXO99.

Supplemental Table S2. The phenotype of *OsDR11S*-suppressing T_0 plants after inoculation with *Xoo* strain PXO99.

Supplemental Table S3. The phenotype of *OsDR11L*-oe T_0 plants inoculated with *Xoo* strain PXO99.

Supplemental Table S4. The phenotype of *OsDR11L-Flag*-oe T_0 plants inoculated with *Xoo* strain PXO99.

Supplemental Table S5. The phenotype of *OsDR11L*-suppressing T_0 plants after inoculation with *Xoo* strain PXO99.

Supplemental Table S6. Primers used for PCR amplification.

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