



## ORIGINAL ARTICLE

# RIR1 represses plant immunity by interacting with mitochondrial complex I subunit in rice

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## Abstract

We previously observed decreased expression of rice OsmiR159a.1 on infection with the bacterial blight-causing pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), and identified the *OsLRR\_RLK* (leucine-rich repeat\_receptor like kinase) gene as an authentic target of OsmiR159a.1. Here, we found that a *Tos17* insertion mutant of *LRR\_RLK* displayed increasing temporal resistance to Xoo, whereas the *LRR\_RLK* overexpression lines were susceptible to the pathogen early on in the infection, indicating that *LRR\_RLK* encodes a repressor of rice resistance to Xoo infection, and it was renamed as *RIR1* (Rice Immunity Repressor 1). *RIR1* overexpression plants were more susceptible to Xoo at late growth stage, suggesting that *RIR1* mRNA levels are negatively correlated with the resistance of rice against Xoo. We discovered that OsmiR159a.1 repression in Xoo-infected plants was largely dependent on the pathogen's type III secretion system. Co-immunoprecipitation, bimolecular fluorescence complementation, and pull-down assays indicated that *RIR1* interacted with the NADH-ubiquinone oxidoreductase (NUO) 51-kDa subunit of the mitochondrial complex I through its kinase domain. Notably, impairment of *RIR1* or overexpression of *NUO* resulted in reactive oxygen species accumulation and enhanced expression of pathogen-resistance genes, including jasmonic acid pathway genes. We propose that pathogens may inhibit OsmiR159 to interfere with the *RIR1*-*NUO* interaction, and subsequently depression of rice immune signalling pathways. The resistance genes manipulated by Xoo can be a probe to explore the regulatory network during host-pathogen interactions.

## KEYWORDS

LRR\_RLK, mitochondrial complex I, OsmiR159a.1, plant immunity, ROS

## 1 | INTRODUCTION

The race to survive between plants and pathogens has been never-ending during the long history of their co-evolution. Plants are

exposed to a wide range of environmental microbes and have evolved sophisticated immune systems to defend against potential pathogens, including two layers of immunity: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity

Lin An, Siyuan Zhang, Rongxiang Fang, and Yantao Jia contributed equally to the work.

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(Jones & Dangl, 2006; Miya et al., 2007; Shiu & Bleecker, 2001). Under normal growth conditions, plant defence pathways are repressed, but they can quickly be activated on pathogen attack. Plant defence responses are involved in complex, interconnected signalling networks that limit a pathogen's spread and access to nutrients (Feys & Parker, 2000), but successful pathogens can overcome plant defences using various strategies, thereby multiplying and causing disease (Gohre & Robatzek, 2008). It is obvious that the final outcome of the interaction between pathogenic organisms and their hosts is determined by the survival strategies employed on both sides.

Accumulating evidence suggests that suppressing RNA-silencing is a common strategy adopted by pathogens to circumvent plant immunity (Ruiz-Ferrer & Voinnet, 2009). Pathogen recognition in compatible plant-microbe interactions is mediated by activation and repression of a large array of genes, and it is evident that host small RNAs are pivotal regulators in reprogramming the expression of these genes (Katiyar-Agarwal & Jin, 2010). Host endogenous sRNAs, including microRNAs (miRNAs), are also integral components in regulating plant responses to various microbes. In turn, pathogenic microbes initiate counterdefences to subvert host cellular silencing pathways (Ruiz-Ferrer & Voinnet, 2009).

Plant receptor-like kinases (RLKs) are a class of transmembrane kinases that structurally resemble the protein kinase receptors of animal cells (Braun & Walker, 1996). RLKs belong to a large gene family with more than 600 and 1100 gene members in *Arabidopsis* and rice, respectively (Shiu & Bleecker, 2003; Shiu et al., 2004). Although several RLKs are reported to function in signalling processes, including recognition of microbial signalling, hormone perception, and developmental regulation (Antolin-Llovera et al., 2012; Morris & Walker, 2003; Shiu & Bleecker, 2001), a large percentage of them await functional characterization. Among them, only a few RLKs with extracellular leucine-rich repeat (LRR) domains are known to play important roles in plant immunity activation (Gomez-Gomez & Boller, 2000; Song et al., 1995; Zipfel et al., 2004), and BRI1 (BAK1-interacting receptor-like kinase 1) was found to be a negative regulator of PTI in *Arabidopsis* (Gao et al., 2009).

Plants as sessile organisms have evolved complex strategies to confront multifarious environmental stresses, including biotic stresses. One strategy employed by plants in pathogen defence is to induce programmed cell death, a process usually initiated and mediated by reactive oxygen species (ROS) (Van Aken & Van Breusegem, 2015). Plasma membranes, chloroplasts, peroxisomes, mitochondria, endoplasmic reticulum, and cell walls are the locations involved in ROS generation (Choudhury et al., 2013). In mitochondria, complexes I and III are the primary redox centres in the mitochondrial electron transport chain (ETC) that can leak single electrons to oxygen and generate ROS formation (Andreyev et al., 2005). Mitochondria are also proposed to be linked with the oxidative burst seen in plant-pathogen interactions. Expression of the HopG1 effector protein in *Pseudomonas syringae* is an example of how plant immunity can be suppressed by a pathogen, in this case by inhibiting mitochondrial function (Block et al., 2010).

The results of our previous study suggested that the coordinated changes in expression of sRNAs in rice in response to blight disease caused by infection with *Xanthomonas oryzae* pv. *oryzae* (Xoo) centred on several sRNAs exhibiting the same expression pattern, which tended to regulate genes in the same or related signalling pathways (Zhao et al., 2015b). We found that this bacterial pathogen can influence a subset of sRNAs and sequentially regulate the genes targeted by sRNAs that may be involved in plant immunity regulation. We identified a novel target gene *LOC\_Os12g10740* of *OsmiR159a.1*, which belongs to the receptor-like kinase gene family (Zhao et al., 2015b), but its function was unknown.

Here, we found that the blight pathogen Xoo manipulates *LRR-RLK* gene expression in rice using its type III secretion system (TTSS). Because an insertion mutant of the *LRR-RLK* gene exhibited increased resistance to Xoo, we called the gene *RIR1* (Rice Immunity Repressor 1). We found that the full-length *RIR1* and *RIR1* kinase domain interacts with a mitochondrial complex I component, NADH-ubiquinone oxidoreductase 51-kD subunit (*NUO*), thereby leading to translocation of *RIR1* to the mitochondria. Overexpression of *NUO* or lack of *RIR1* increased ROS accumulation and pathogen resistance-marker gene expression in rice leaves. Our findings indicate that immune activation of the *rir1* mutant is partially caused by an increase in ROS, and Xoo may hijack the host immune system by restricting ROS production in mitochondria.

## 2 | RESULTS

### 2.1 | Down-regulation of *OsmiR159a.1* by the bacterial TTSS is inversely related to *RIR1* expression

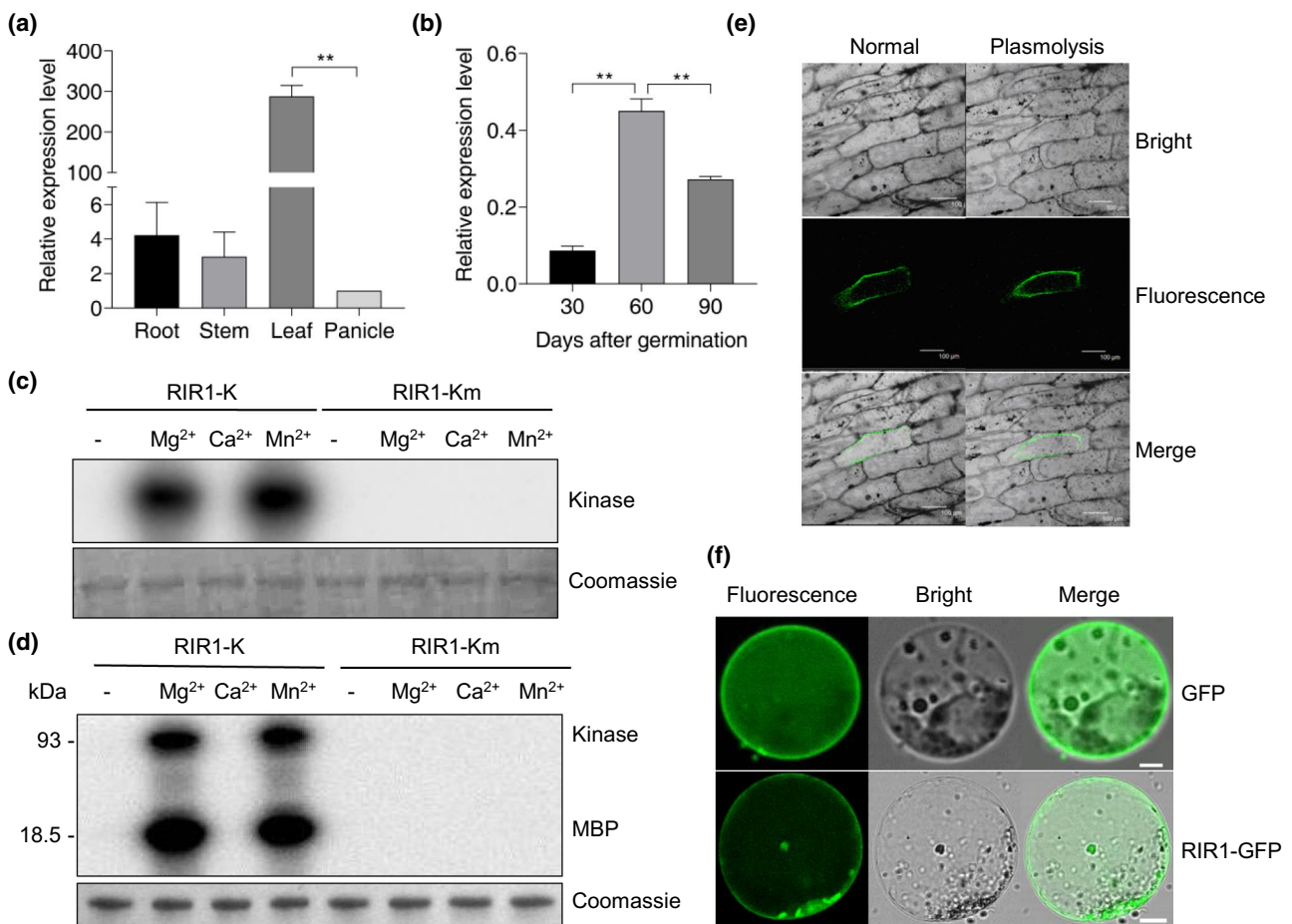
Successful pathogens use the TTSS to deliver diverse effector proteins into host plant cells, thereby manipulating plant immunity, which leads to dynamic changes in miRNA levels in the infected plants (Pumplin & Voinnet, 2013). Our previous study found that the level of *OsmiR159a.1* was reduced at 24 h postinoculation with the Xoo PXO99 (race 6) strain. To investigate whether the expression of *OsmiR159a.1* is influenced by the TTSS of Xoo, the leaves from 60-day-old rice (*Oryza sativa* subsp. *japonica* 'Nipponbare') were challenged with the wild-type (WT) Xoo strain or the TTSS-deficient mutant strain,  $\Delta hrcU$ , and double-deionized water (ddH<sub>2</sub>O) treatment as mock control. As shown in Figure S1a, mature *OsmiR159a.1* level was significantly higher in  $\Delta hrcU$  mutant-inoculated leaves than those in the WT-inoculated leaves at 24 h after bacterial infection as measured by northern blotting. In contrast, the *OsmiR159a.1*-targeted *RIR1* gene's expression level was lower in the  $\Delta hrcU$ -inoculated rice leaves than in the WT Xoo-infected leaves, as measured by reverse transcription-quantitative PCR (RT-qPCR) (Figure S1b). Thus, the Xoo TTSS may play a role in pathogenicity via targeting *RIR1* mRNA by regulating *OsmiR159a.1* in the host rice plant.

## 2.2 | *RIR1* encodes a plasma membrane-associated kinase and has different expression patterns in different tissues

To examine the expression pattern of *RIR1*, RT-qPCR was performed using total RNA extracted from various tissues. Comparison of the *RIR1* expression in different tissues revealed that the *RIR1* transcripts were highly abundant, but the *RIR1* transcript amounts were mostly lower in the roots and stems, and extremely low in the panicles (Figure 1a). We also quantified the *RIR1* mRNA expression levels in leaves during their different growth stages by RT-qPCR and found that the *RIR1* mRNAs were most abundant at 60 days after germination (Figure 1b). These results suggest that *RIR1* regulation is tissue-specific and aging-dependent.

The C-terminal region of *RIR1* (amino acids 639–908) contains a putative protein kinase catalytic domain, as determined by SMART (Simple Modular Architecture Research Tool) prediction, and with an RD motif in subdomain VIb, *RIR1* is considered to be an RD kinase (Figures S2 and S3). The presumed extracellular domain contains LRRs (as predicted by the LRR finder) (Offord & Werling, 2013), which are known to be involved in protein–protein interactions. Each unit of the LRR repeats contains the LXXLX<sub>n</sub>NXFXXG(L/I)PXX(L/I)GXLXX consensus sequence (Figure S2). One predicted transmembrane region is linked to the LRR repeats and kinase domain (Figure S2).

Histidine-tagged *RIR1* cytoplasmic kinase domain was designed to enable its soluble expression in *Escherichia coli* and, as shown in Figure 1c, the *RIR1* kinase domain displayed autophosphorylation activity. The kinase domain sequence alignment for *RIR1*,



**FIGURE 1** The temporal-spatial expression pattern of *RIR1* in rice tissues, *RIR1* subcellular localization, and its kinase domain activity. (a) *RIR1* expression was assayed in root, stem, leaf, and panicle tissues. *RIR1* levels in leaves were the highest among the tested tissues. Relative expression level of *RIR1* determined by reverse transcription quantitative PCR (RT-qPCR). The expression level of *RIR1* in panicle is indicated as 1.0. Rice *actin* was used as the internal reference gene for normalization. (b) The expression level of *RIR1* gene at different days after germination. Error bar represents SE for at least three replicates with statistical analysis using one-way analysis of variance (ANOVA) test (\*\* $p < 0.01$ ). (c) *RIR1* kinase domain (*RIR1*-K) autophosphorylation depends on Mg<sup>2+</sup> and Mn<sup>2+</sup>, but not Ca<sup>2+</sup>. The key amino acid substitution of R827G (*RIR1*-Km) leads to the disruption of kinase activity. The purified protein was resolved by SDS-PAGE to examine the loading after Coomassie blue staining. (d) The *RIR1* kinase domain can phosphorylate myelin basic protein (MBP) depending on Mg<sup>2+</sup> and Mn<sup>2+</sup>, but not Ca<sup>2+</sup>. (e) The *RIR1*-GFP fusion protein was transiently expressed in onion epidermal cells and visualized by confocal microscopy. The same cell was photographed before and after plasmolysis. Scale bar, 100 μm. (f) Confocal images showing the *RIR1*-green fluorescent protein (GFP) fusion protein localized at plasma membrane and cytoplasm in rice protoplast cells. As a control, GFP alone was transiently expressed in rice cells using plasmid pBI221-*RIR1*-GFP. Scale bar, 7.5 μm

BRI1 (Li & Chory, 1997), CLV1 (Clark et al., 1997), and Xa21 (Song et al., 1995) shows that these kinases contain several conserved motifs (Figure S3). RIR1 contains HRDIKSSN in subdomain VIb and GTMGYLDPEY in subdomain VIII, indicating that it may function as a serine/threonine kinase (Hanks & Quinn, 1991). The YSFGVLL motif is highly conserved among several putative LRR receptor kinases (Hanks & Quinn, 1991; Li & Chory, 1997), which implies it has an essential function. To determine the importance of one of the conserved amino acids for the catalytic activity of RIR1, a mutant protein where glycine was substituted with arginine (G827R) was generated. The resulting recombinant RIR1 kinase protein displayed impaired kinase activity, leading to its failure to autophosphorylate and for MBP (Myelin Basic Protein, a substrate for kinase assay) phosphorylation *in vitro* (Figure 1d). However, RIR1 kinase activity was stimulated by the presence of 10 mM Mg<sup>2+</sup> or Mn<sup>2+</sup>, but not Ca<sup>2+</sup>.

Obtaining information about the subcellular localization of a protein is important if we are to understand a protein's function. Therefore, to examine the subcellular localization of RIR1, we constructed recombinant protein with the cauliflower mosaic virus (CaMV) 35S promoter (p35S) and RIR1-green fluorescent protein (GFP). Transient expression experiments with RIR1-GFP were performed in onion epidermis cells and rice protoplast cells. As shown in Figure 1e,f, RIR1-GFP was mainly located at the plasma membrane. GFP alone was also transfected as a control.

### 2.3 | RIR1 plays a negative role in plant immunity

Besides the gibberellin-encoding gene (*GAMYB*), *RIR1* is another verified target of OsmiR159a.1 (Zhao et al., 2015b). To more precisely distinguish the function of *RIR1* from those of other potential targets of OsmiR159a.1, we investigated whether a correlation exists between the pathogen-resistance phenotype and the expression level of *RIR1*. The *RIR1* retrotransposon *Tos17* insertion mutant plant (NF9013) (*rir1* mutant) contains an insertion in the promoter region. The *rir1* mutant plants were found to be more resistant to Xoo compared with the 60-day-old WT plants (Figure 2a,b), and no *RIR1* mRNA or protein expression was detected in these mutants (Figure 2c,d). To construct a plant that overexpresses *RIR1*, the *RIR1* genomic DNA fragment driven by the CaMV 35S promoter was cloned into a binary vector and introduced into rice. The same backbone was used to produce the p35S::*RIR1* cDNA construct, but *RIR1* mRNA was not detected in the transgenic plants for some unknown reason. The *RIR1* overexpression (*RIR1* OE) plants and WT rice plants (60 days old) showed similar levels of resistance to Xoo (Figure 2a,b), indicating that even higher than normal transcriptional and translational levels of *RIR1* did not make the rice more susceptible to bacterial infection (Figure 2a,c,d).

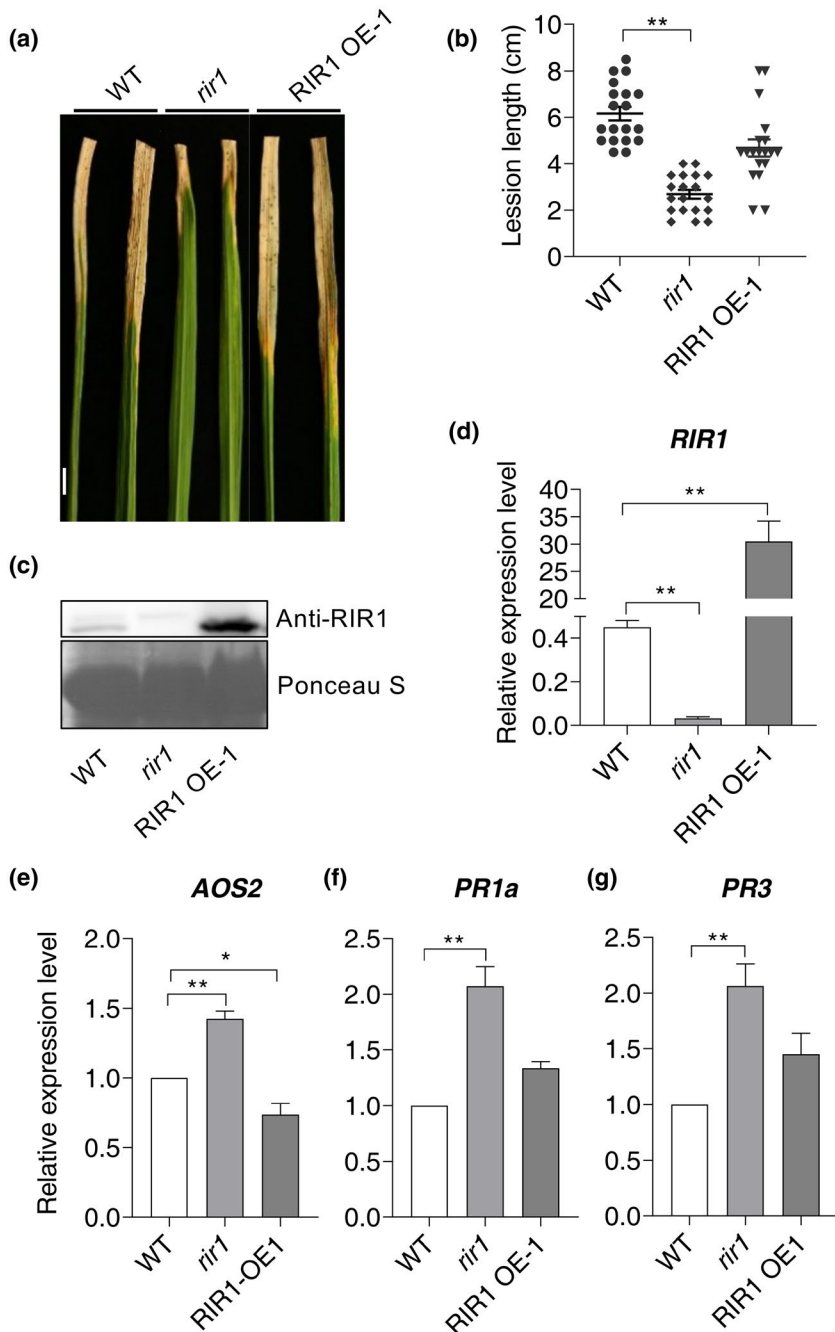
To further investigate how *RIR1* is involved in regulating gene expression during the plant immune response, the levels of a set of defence-responsive genes including *PAL1* (phenylalanine ammonia-lyase 1 gene), *PAD4* (phytoalexin-deficient 4 gene), *ICS1* (isochorismate synthase 1 gene), *AOS2* (allene oxide synthase 2 gene), *NH1* (*Arabidopsis*

NPR1 homolog 1 gene), *LOX2* (lipoxygenase 2 gene), *PLDβ1* (phospholipase Dβ1 gene), *PR1a* (pathogenesis-related protein 1A gene), *PR3* (pathogenesis-related protein 3 gene), *PR5* (pathogenesis-related protein 5 gene), and *PR10* (pathogenesis-related protein 10 gene) were quantified by RT-qPCR after Xoo inoculation. When the rice leaves were challenged with Xoo, most of the tested genes showed no obvious changes (Figure S4), but the mRNA expression levels of *PR1a* and *PR3* were up-regulated about twofold, and *AOS2* mRNA expression significantly increased in the *rir1* mutant compared with that of the WT plants at 16 h postinfection (hpi) (Figure 2e–g). *RIR1* OE led to a slightly lower level of *AOS2* mRNA, whereas the levels of the other two genes' mRNAs showed no obvious differences from those of the WT plants (Figure 2e–g).

### 2.4 | RIR1 directly interacts with the complex I subunit via its kinase domain

To further unravel the components interacting with RIR1 in rice, we selected rice overexpressing *RIR1* with which to perform a co-immunoprecipitation (Co-IP) coupled liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Co-IP of the cell lysates using anti-RIR1 antibodies was performed to identify potential interacting proteins. The LC-MS/MS analysis revealed that one set of abundant peptides in the tryptic digests was derived from the NADH-ubiquinone oxidoreductase 51 kDa subunit (designated NUO) (LOC\_Os07g45090.1), which is a component of the respiratory chain's mitochondrial complex I. *Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* GV3101 containing genes encoding p35S::NUO-HA along with either p35S::RIR1-Myc or p35S::RIR1-N-Myc/RIR1-K-Myc. The interaction between RIR1 and NUO was confirmed by Co-IP assay. As shown in Figure S5, the full-length RIR1 and RIR1 kinase domain interacted with NUO. Because the full-length cDNA-expressed RIR1 was found in the insoluble inclusion bodies from *E. coli* cells, a truncated RIR1 protein containing the kinase domain was used to confirm the association between RIR1 and NUO by a pull-down experiment, using maltose-binding protein (MBP)-tagged RIR1 kinase domain (MBP-RIR1-K) as the bait and His-tagged NUO (His-NUO) as the prey. Amylose resin pull-down and immunoblotting showed retention of MBP-RIR1 along with His-NUO but not with the MBP control, indicating that the direct interaction between RIR1 kinase domain and NUO exists (Figure 3a).

We also conducted bimolecular fluorescence complementation (BiFC) assays to test whether the kinase domain from RIR1 could interact with NUO in rice protoplasts. The RIR1 kinase domain and NUO were fused to the N-terminal of the yellow fluorescent protein (YFP) (RIR1-K-nYFP) and the C-terminal of YFP (NUO-cYFP), respectively. As expected, when the two constructs were cotransformed into rice protoplast cells, YFP-specific fluorescence was observed in the mitochondria, but BiFC fluorescence was not observed in the RIR1-K-nYFP/cYFP or NUO-cYFP/nYFP pairs (Figure 3b), indicating that RIR1 kinase can shift its subcellular localization into mitochondria by binding to NUO. To further confirm this conclusion, the RIR1



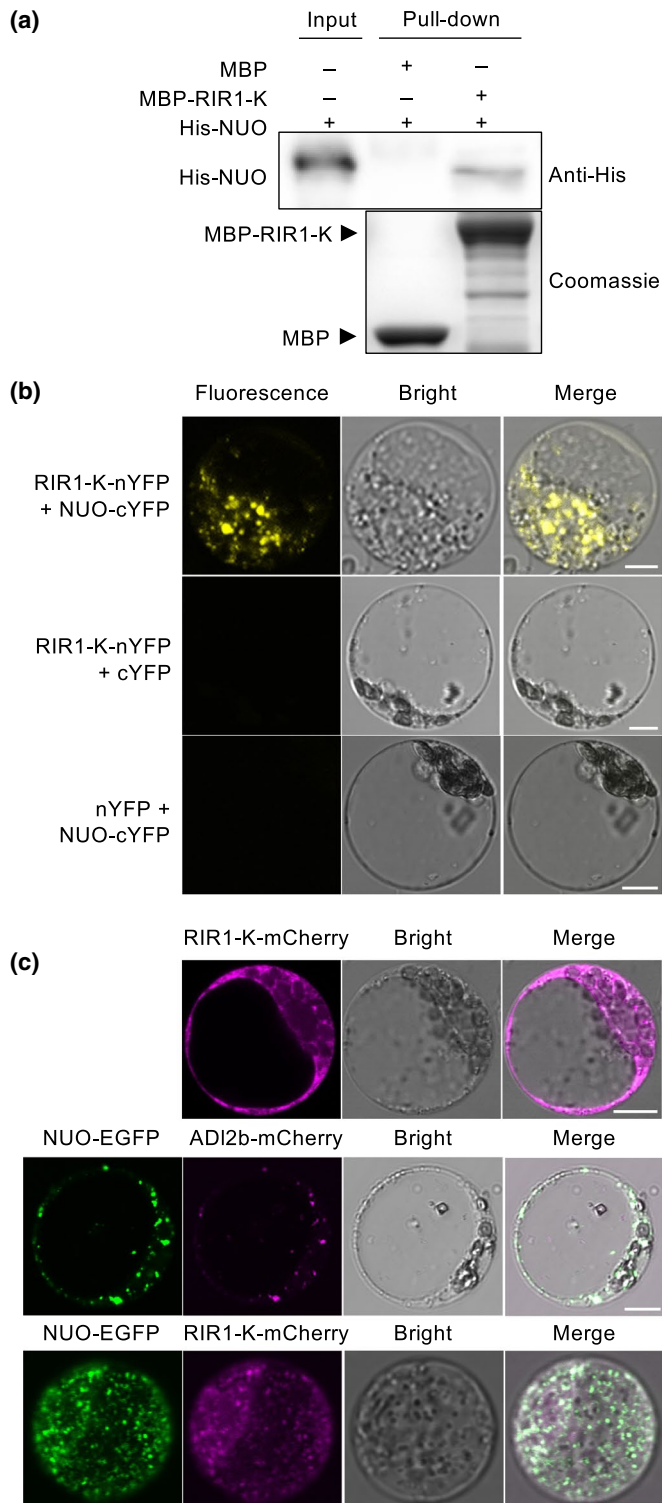
**FIGURE 2** RIR1 is a negative regulator of the plant immune system. (a) Disease phenotypes of wild-type (WT), *rir1*, and RIR1 overexpression (OE)-1 plants at 14 days after *Xanthomonas oryzae* pv. *oryzae* (Xoo) infection. Scale bar, 1 cm. (b) The lesion length was measured at 14 days postinoculation with Xoo. The leaves of RIR1 null mutant *rir1* displayed reduced lesion lengths after Xoo inoculation. The lesion lengths in the RIR1 OE plants were not significantly different to those of the control plants. Error bars represent the SE of at least three replicates and one-way analysis of variance (ANOVA) test was used (\*\* $p < 0.01$ ). (c) Western blotting analysis of RIR1 expression levels. The protein level in RIR1 OE plants was higher than that of the WT. RIR1 is absolutely not expressed in *rir1*. The Ponceau S staining served as a loading control. (d) Expression levels of RIR1 in WT, *rir1*, and RIR1 OE-1 plants. Error bars represent SE for at least three replicates with statistical analysis using Student's *t* test (\*\* $p < 0.01$ ). (e–g) Relative expression of AOS2, PR1a, and PR3 in WT, *rir1*, RIR1 OE-1 plants. The expression level of each gene in WT is indicated as 1.0. Rice *actin* was used as the internal reference gene in reverse transcription quantitative PCR (RT-qPCR). Error bars represent SE for at least three replicates and one-way ANOVA was used (\*\* $p < 0.01$ , \* $p < 0.05$ )

kinase domain and NUO were fused with mCherry and enhanced GFP (eGFP) to generate RIR1-K-mCherry and NUO-eGFP, respectively. When we separately expressed the two constructs in rice protoplast cells, RIR1-K-mCherry produced uniform fluorescence throughout the cytoplasm, and NUO-eGFP was visualized in the mitochondria (the NUO-eGFP and ADL2b-mCherry mitochondrial markers with completely matching images are shown in Figure 3c), but co-expression of the two fusion proteins led to the eGFP and mCherry fluorescence colocalizing in the mitochondria. Immunoblotting analyses of subcellular fractions in RIR1 and NUO transiently expressed *N. benthamiana* confirmed that RIR1 and NUO localized in the cytoplasmic fractions (Figure S6a). In RIR1 overexpression rice plants, the full-length RIR1 localized in both cytoplasm and plasma membrane (Figure S6b). These

results are consistent with the BiFC assay and Co-IP assays, supporting the conclusion that the RIR1 interacts with NUO in vivo.

## 2.5 | Elevated expression of NUO promotes plant immune responses

In mitochondria, NADH-ubiquinone reductase (complex I) is one of the redox centres in the mitochondrial ETC that releases electrons to molecular oxygen to produce superoxide (Andreyev et al., 2005). To investigate the function of NUO in plant immunity, we chose four target sites from its coding regions (CDS) to generate novel NUO variants using the CRISPR/Cas9 system (Figure S7). Breeding of the heterozygous rice



**FIGURE 3** Interaction between the RIR1 kinase domain and NUO. (a) In vitro pull-down assay of His-NUO with the bait protein maltose-binding protein (MBP)-RIR1-K or MBP. Top panel: anti-His western blot analysis revealed the presence of His-NUO in the input lysate and in affinity pulldown capture. Bottom panel: MBP-RIR1-K bait protein after MBP pulldown. (b) Bimolecular fluorescence complementation (BiFC) assay shows the interaction between RIR1-K and NUO in rice protoplasts. Scale bar, 7.5  $\mu$ m. (c) Colocalization of NUO-eGFP fusion protein and RIR1-K-mCherry fusion protein in rice protoplast. Scale bar, 7.5  $\mu$ m

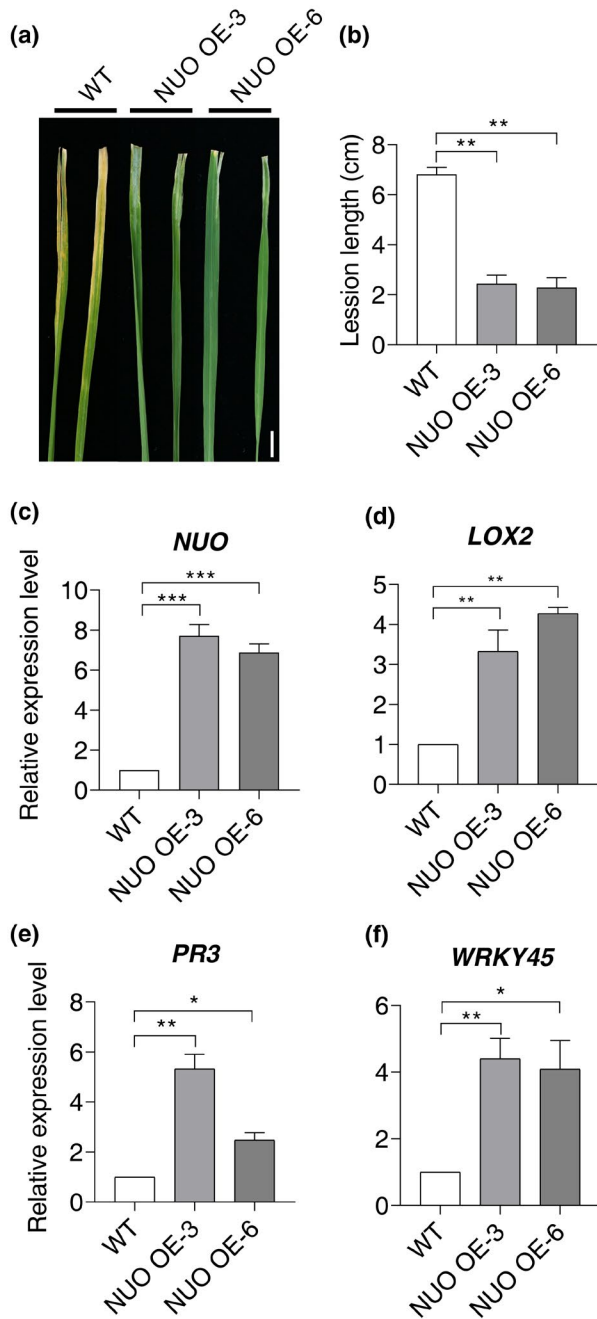
plants for three generations ( $T_1$ - $T_3$ ) led to the generation WT and heterozygous lines, but unfortunately no homozygous line was obtained, strongly suggesting that mutating NUO was lethal at an early developmental stage. Plants overexpressing NUO were generated by introducing the construct containing the full-length NUO cDNA fragment with the 35S promoter into the Nipponbare rice cultivar. When transgenic 60-day-old plant leaves were challenged with Xoo, the transgenic NUO overexpression (NUO OE) plants were more resistant to Xoo than the WT rice. The average lesion length in the NUO OE plants was 2.4 cm, compared with 6.8 cm for the WT control at day 14 after inoculation (Figure 4a,b). To confirm that resistance in the transgenic plants was caused by NUO OE, the level of NUO in the NUO OE transgenic plants was examined. As shown in Figure 4c, the mRNA level of NUO in the NUO OE plants was significantly higher than in WT plants. Consistent with the resistance phenotype, *LOX2*, *PR3*, and *WRKY45* mRNA levels significantly increased in NUO OE compared with those in the WT after Xoo inoculation (Figure 4d,e,f). Among them, the jasmonate (JA) synthesis gene *LOX2* was up-regulated about 4-fold in the NUO OE lines as compared with the WT levels (Figure 4d).

## 2.6 | Regulating ROS generation influences immune responses

Biotic stresses can cause alterations in ROS production in plant cells, and these molecules are critical for priming an effective defence response against bacterial pathogens (Apel & Hirt, 2004; Torres et al., 2002; Wen et al., 2008). Mitochondria are considered to be the main sources of ROS generation, and two primary redox centres in the mitochondrial respiratory chain, complexes I and III, are the major sites of ROS production (Andreyev et al., 2005). The NADH-ubiquinone oxidoreductase 51-kDa subunit (or NUO) is a subunit of mitochondrial complex I. We detected ROS production using a luminol-based extracellular ROS assay in rice leaves with flg22 acting as the elicitor. As shown in Figure 5a, NUO OE plants showed higher levels of ROS than WT plants, with the peak ROS level appearing 12 min after flg22 treatment. We also observed that ROS accumulation was higher in the *rir1* mutant than in the WT plants, with the peak ROS level observed 16 min after adding flg22, whereas the RIR1 OE line did not cause significant alterations in ROS production (Figure 5b). To test whether RIR1 suppresses ROS by interaction with NUO, we tried to make a cross between the NUO OE and *rir1* mutant plants, but failed to get hybrid seeds.

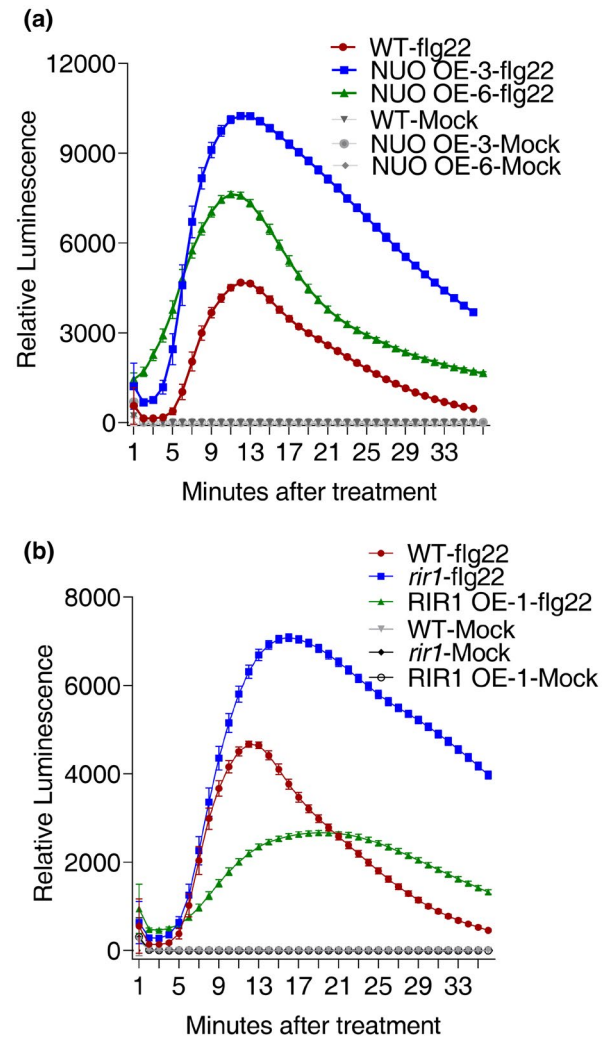
## 3 | DISCUSSION

Plants trigger diverse defence reactions against invading pathogens; endogenous sRNAs are believed to be important plant defence regulators, and specific miRNAs have been shown to suppress PTI during pathogen infections (Ruiz-Ferrer & Voinnet, 2009). Here, we found that OsmiR159a.1 levels were induced by Xoo TTSS-deficient mutant strain  $\Delta$ *hrcU* compared with that of Xoo WT treatment. The miR159 family is one of eight highly conserved miRNA families in



**FIGURE 4** NUO promotes plant immune responses. (a) Disease phenotypes of wild-type (WT), NUO overexpression (NUO OE) plants at 14 days after infection with *Xanthomonas oryzae* pv. *oryzae* (Xoo). Scale bar, 1 cm. (b) The lesion length was measured at 14 days after Xoo infection. (c–f) Relative expression levels of NUO, LOX2, PR3 and WRKY45 in WT, NUO OE plants. The expression level of the target gene in WT plants is indicated as 1.0. *Actin* was used as the internal reference gene in reverse transcription quantitative PCR (RT-qPCR). Error bar represents SE for at least three replicates and one-way analysis of variance (ANOVA) test was used (\*\* $p < 0.01$ , \* $p < 0.05$ )

the common ancestor of all embryophytes, and forms an indispensable component of functional regulatory networks in such plants (Cuperus et al., 2011). In *Arabidopsis*, miR159 is predicted to regulate



**FIGURE 5** Reactive oxygen species (ROS) accumulation in rice leaves after flg22 treatment. (a) A flg22-induced ROS burst in wild-type (WT) and NUO overexpression (OE) plants. (b) A flg22-induced ROS burst in WT, *rir1*, and RIR1 OE plants. Rice leaf disks were treated with 1  $\mu$ M flg22 or water. Error bar represents the SE of at least three replicates

more than 20 target genes (Millar et al., 2019; Palatnik et al., 2007). OsmiR159a.1 has multiple targets in rice, as deduced by target prediction algorithms, and two targets of OsmiR159a.1, *GAMYB*, and *RIR1* (LOC\_Os12g10740) we validated previously (Zhao et al., 2015b). We believe that exploring the function of miRNA-target genes is an essential first step in the discovery of plant defence mechanisms and could be an important strategy for mining new resistance-related genes.

The predicted product encoded by *RIR1* has a structure typical of LRR receptor kinase-like proteins (LRR-RLKs), whose functions are uncharacterized. Pfam analysis showed that *RIR1* belongs to the monophyletic interleukin-1 receptor-associated kinase family, LRR-VIII-1 subfamily. Here, the lack of *RIR1* led to enhanced disease resistance to the Xoo bacterial pathogen (Figure 2a,b). The *RIR1* expression level in 90-day-old plant leaves was significantly lower than

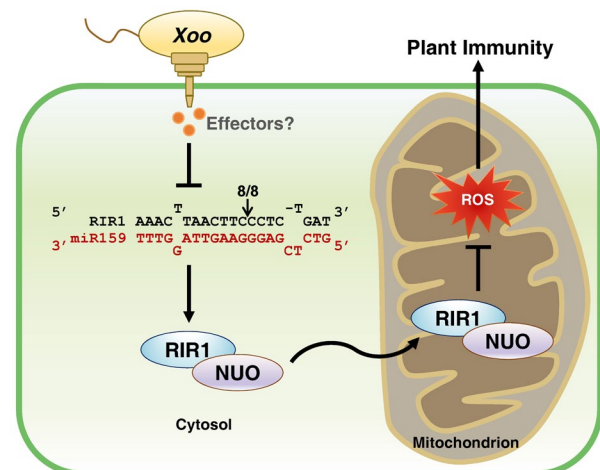
that in 60-day-old plant leaves. In fact, the *RIR1* OE lines were more sensitive to *Xoo* compared to the WT line during the late growth stages (Figure S8a), and the mRNA levels of the resistance marker genes were much lower in the *RIR1* OE line compared with the WT plants (Figure S8b,c). It is likely that *RIR1* expression is developmentally controlled and, as seen in many plant–pathogen interactions, as a plant matures, its resistance level continues to increase (Cao et al., 2007). These results indicate that the increased resistance of rice can be partially explained by the low level of *RIR1*, and *RIR1* might play an important role in coordinating the balance between rice development and biotic resistance.

The C-terminal kinase domain of RLK when fused to the N-terminal LRR domain always exerts its function in signal transduction cascades wherein extracellular signals are transmitted through the cell membrane to the cytoplasm to modify gene expression (Couto & Zipfel, 2016). Consistent with previous research findings, as an RD kinase, *RIR1* can either phosphorylate itself or artificial substrate proteins (Figure 1c,d). It has been reported that most kinases associated with PTI replace the positively charged R in the catalytic loop with uncharged residues, the exception being the *Arabidopsis* chitin elicitor receptor kinase, CERK1 (Miya et al., 2007). *RIR1* is another RLK-containing RD kinase involved in plant immune responses. We found that *RIR1* is associated with the 51-kDa NUO subunit, which is one of the core subunits in the respiratory chain complex I. We deduce that elevated levels of *RIR1* may allow *RIR1* to interact with NUO during the biogenesis processes in the cytoplasm. As shown in Figure S6, both *RIR1* and NUO could be detected in cytoplasm. Mis-localization of the *RIR1* kinase domain was observed, leading to the *RIR1* kinase shifting from the cytoplasm to the mitochondria when co-expressed with NUO (Figure 3b,c). One possibility is that the mitochondrial NUO protein may be modified by binding to *RIR1*, thereby affecting ROS mitochondrial homeostasis. It is reported that superoxide produced in the mitochondrial matrix is quickly dismuted to  $H_2O_2$  and readily diffuses out of mitochondrial membranes (Dikalov & Harrison, 2012). NUO is the large subunit of complex I (the main ROS generation site in mitochondria); disturbing NUO can lead to production of ROS, which may function as signal molecules to contribute to triggering ROS generation in different organelles or compartments of the cell. Cascade network and direct communication from chloroplasts to mitochondria have been demonstrated previously (Zhao et al., 2018).

Dysfunction of mitochondrial complex I leads to over-reduction of the mitochondrial ETC, which causes higher mitochondrial ROS production (Møller, 2001). We found that the complex I NUO subunit mutation in rice may lead to a lethal effect, suggesting that NUO is critical for rice growth and development. We found that *NUO* OE lines displayed increased ROS levels by responding to flg22 treatment (Figure 5a), and a lack of *RIR1* led to ROS accumulation in rice. *RIR1* may be important for helping plants repress overaccumulation of ROS by interfering with mitochondrial complex I. Two genes in the jasmonic acid (JA) biosynthesis pathway, *LOX2* (encoding lipoxygenase) and *AOS2* (encoding allene oxide synthase), were regulated by *RIR1* and NUO. The impairment of

*RIR1* enhanced the expression of *AOS2* (Figure 2e), and overexpression of *NUO* led to increased expression of *LOX2* (Figure 4d). This suggests that *RIR1* and *NUO* each play a role in modulating JA biosynthesis in rice, which is consistent with previous findings that JA confers resistance to pathogen infections (Yamada et al., 2012; Zhao et al., 2015a; Zhu et al., 2014). Salicylic acid (SA) plays a role in defence responses against biotrophic pathogens (such as *Xoo*), but SA synthesis and signalling pathway genes were not altered by *RIR1* and *NUO*. It has been reported that *PR3* is responsive to JA during rice–*Xoo* interactions (Pang et al., 2013). We found that *PR3* expression levels increased in the *rir1* mutant and *NUO* OE lines alike, suggesting that the regulating cascade may be related to the JA signalling pathway.

We have prepared a working model to illustrate the relationships among the related components that regulate plant immunity (Figure 6). The physiological significance of *RIR1* for the host plant is that it might make the plant maintain a relatively low level of resistance during growth and development. Virulent pathogens such as *Xoo* use the TTSS to overcome immune responses in the host plant by inducing *RIR1* expression by repressing *OsmiR159a.1*. Subsequently, elevated *RIR1* associates with *NUO* to limit ROS production, thereby subverting plant immunity to favour invading plant pathogens. Plant resistance comes with a negative fitness cost; hence, resistance alleles have not been driven to fixation by natural selection, making it hard to identify novel pathogen-resistance genes. This study provides a useful method for probing resistance-related genes by investigating the sRNA targets regulated by their cognate pathogens. Notably, loss of pathogen susceptibility via inhibition of a negative-resistance regulator, such as *RIR1*, to generate durable pathogen-resistant plants could be a useful strategy to improve agricultural breeding.



**FIGURE 6** Working model for pathogen interference with plant immunity in rice. The bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* may use unknown effector proteins to down-regulate the expression of *OsmiR159a.1*, and one target of *OsmiR159a.1*, *RIR1*, is up-regulated. Elevated *RIR1* binds to *NUO* and translocates into mitochondria, thereby inhibits reactive oxygen species (ROS) generation and represses plant immunity



## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Plant materials and growth conditions

All WT rice (*O. sativa* subsp. *japonica* 'Nipponbare') and transgenic plant seeds were sown, field-grown for 1 month, and then transferred and planted apart under natural conditions until harvesting. The *Tos17* insertion mutant (NF9013) was provided by the National Institute of Agrobiological Sciences (NIAS), Japan (<http://www.rgrc.dna.affrc.go.jp>). All of the mutant and transgenic lines listed above are derived from Nipponbare. The *Tos17* primer and two flanking primers (Flank-F and Flank-R) were used to ensure that *Tos17* was inserted into the potential promoter region of *LOC\_Os12g10740*. The primer sequences are listed in Table S1.

### 4.2 | Pathogen inoculation

Leaves from 60-day-old rice plants were inoculated with the Xoo strain PXO99 (Philippine race 6) using the leaf-clipping method with scissors dipped in the bacterial suspension ( $OD_{600} = 0.5$ ). The Xoo strain was grown overnight, washed twice, and then resuspended in sterile distilled water. Lesion length was measured at 14 days after inoculation.

### 4.3 | Transgenic plant generation

The 7.4 kb full-length *RIR1* genomic sequence was PCR-amplified with primers LRR-OE-1-F and LRR-OE-3-R, and the 1.5 kb full-length *NUO* coding sequence was amplified from rice with primers NUO-OE-F and NUO-OE-R, and then subcloned into the binary pCAMBIA1300 vector to create pCAMBIA1300-RIR1 and pCAMBIA1300-NUO, respectively, with *RIR1* and *NUO* under the control of the CaMV 35S promoter.

The above constructs were introduced into *A. tumefaciens* EHA105 and then transformed into Nipponbare. The rice callus transformants were screened on plates containing 50  $\mu\text{g}/\text{ml}$  hygromycin.

### 4.4 | CRISPR/Cas9-mediated gene editing

Nipponbare rice was genetically modified with CRISPR/Cas9 as described previously (Meng et al., 2017). Briefly, target sequences were selected within the exon region of the *NUO* genomic sequence, and four sgRNAs (*NUO-a*, *NUO-b*, *NUO-c*, and *NUO-d*) were designed with CRISPR direct software (<http://crispr.dbcsls.jp/>). The sgRNA/Cas9 constructs were transferred into Nipponbare callus by *Agrobacterium*-mediated transformation. Genomic DNA from the CRISPR/Cas9-edited rice leaves was used to PCR-amplify the target region with specific primers (Table S1). The resulting amplicons were used to confirm the mutation by direct sequencing.

### 4.5 | Mass spectrometric analysis screening of RIR1-interacting proteins

To identify RIR1-interacting proteins, leaves (2 g) from *RIR1* OE plants or *rir1* plants were ground in liquid nitrogen. The powder was resuspended in 4 ml of co-immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.2% NP-40, 6 mM  $\beta$ -mercaptoethanol, 1 $\times$  protease inhibitor cocktail). Debris was removed from the lysate by centrifugation at 15,000  $\times$  g. The lysate was incubated with the protein G-RIR1 antibody complex for 4 h and, after washing with co-immunoprecipitation buffer six times, the sample was eluted with 30  $\mu\text{l}$  of 50 mM glycine solution according to the Dynabeads Protein G manual's instructions (Thermo Fisher). The eluted proteins were loaded into a single lane for 10% SDS-PAGE. Protein bands on the gel were trypsin-digested (10 ng/ml trypsin, 50 mM ammonium bicarbonate pH 8.0) at 37°C overnight. Peptide analysis by LC-MS/MS was performed by the Protein World Biotech Company.

### 4.6 | Gene expression analyses

To examine the influence of Xoo infection on rice gene expression, 0.5-cm leaf fragments next to the bacterial infection sites were used for RNA isolation. Total RNA was extracted from the fragments with TRIzol reagent (Invitrogen Life Technologies). A 2  $\mu\text{g}$  aliquot of total RNA pretreated with DNase I was reverse-transcribed using SuperScript III (Invitrogen) and oligo(dT<sub>23</sub>) primers. qPCR was performed to quantify a given cDNA transcript amount using SYBR Green PCR master mix (Applied Biosystems) and gene-specific primers (Table S1). The *actin* gene was used as the internal control.

To analyse the expression of *OsmiR159a.1*, 30-day-old rice leaves were scissor-clipped with bacterial suspensions ( $OD_{600} = 1.00$ ) of Xoo and the  $\Delta hrcU$  mutant. About 20  $\mu\text{g}$  of total RNA from each sample was resolved on a 17.5% polyacrylamide-urea gel, electrotransferred to Biotrans nylon transfer membranes (PALL Life Sciences), and an antisense oligonucleotide probe was used for hybridization to *OsmiR159a.1*.

### 4.7 | Subcellular localization

The *RIR1* coding sequence was PCR-amplified from Nipponbare cDNA using RIR1CDS-GFP-F and RIR1CDS-GFP-R primers, and the resulting gene fragment was subcloned into plasmid pBI221-GFP to generate pBI221-RIR1-GFP, with GFP fused in frame to the 3' end of the *RIR1* gene.

For the GFP assay, 1  $\mu\text{g}/\mu\text{l}$  of pBI221-RIR1-GFP was introduced into onion epidermal cells following the PDS-1000/He Biolistic Particle Delivery System's (Bio-Rad) protocol under the conditions of 1100 psi, shelf NR.5 (9 cm), 27 inches of Hg vacuum. Next, onion epidermal cells were cultured on Murashige & Skoog (MS) plates

and incubated at room temperature overnight in the dark. To investigate *RIR1* expression in rice, 1  $\mu\text{g}$  of plasmid pBI221-RIR1-GFP was transfected into rice protoplasts using the polyethylene glycol 400-mediated transfection method described previously (Chen et al., 2006). Both onion epidermal cells and rice protoplasts were visualized by laser scanning confocal microscopy (TCS SP8; Leica). To distinguish between the cell wall and plasma membrane, the onion epidermal cells were treated with 30% sucrose solution for 3 min to induce plasmolysis.

#### 4.8 | BiFC assays

BiFC assays were performed as described previously (Bart et al., 2006). The coding regions of the NUO and RIR1 kinase domains were inserted into puc-SPYCE or puc-SPYNE, respectively (Waadt et al., 2008). The recombinant constructs were co-expressed in rice protoplasts, then observed by confocal laser scanning microscopy.

The subcellular locations of the individual NUO and RIR1 kinase domains were determined according to Bart et al. (2006). For protein subcellular localization detection, the coding sequences were cloned into pBI221 to generate pmCherry-RIR1-K or peGFP-NUO for NUO or RIR1, respectively. The fluorescence signals from the fusion proteins in the rice protoplasts were observed by confocal laser scanning microscopy.

#### 4.9 | Vector construction, antibody preparation, and western blotting

The RIR1 kinase domain-encoding DNA sequence was PCR-amplified and cloned into the prokaryotic expression vector pET30a to generate pET30a-RIR1K for expression in *E. coli* BL21 (DE3) cells. The purified recombinant protein was used as an antigen to immunize a rabbit for polyclonal antibody production by Invitrogen.

Total proteins were extracted from the rice leaves with extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% vol/vol glycerol, 0.1% vol/vol NP40, and cocktail protease inhibitor), and then separated on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Pall Corporation). Each membrane was blocked in blocking buffer (5% skimmed milk, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% vol/vol Tween 20), and then incubated in blocking buffer containing the diluted primary antibody (1:5000, 2 h, room temperature). The primary antibody solution was added and the membrane was washed three times with TBST buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% vol/vol Tween 20), and then incubated in blocking buffer containing a 1:10,000 dilution of horseradish peroxidase-linked secondary goat anti-rabbit antibody (1 h, room temperature) and washed three times. Immunodetection was performed using the EZ-ECL HRP Substrate Kit (Biological Industries).

#### 4.10 | Pull-down assays

Recombinant His-NUO, MBP or MBP-RIR1-K were expressed in *E. coli* BL21 (DE3) for use in the pull-down assays. Co-precipitation of NUO containing the RIR1 kinase domain was examined by western blotting after affinity purification using amylose resin (NEB) following the manufacturer's instructions. Total proteins were extracted in binding buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 $\times$  protease inhibitor cocktail [Roche]) at 4  $^{\circ}\text{C}$ , and then coprecipitated with NUO with RIR1-K in the binding buffer. After incubation, the resin was washed five times with washing buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 $\times$  protease inhibitor cocktail [Roche]) and eluted in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM amylose. The presence of His-NUO in the eluate was detected with an anti-HIS antibody (Abmart).

#### 4.11 | *Agrobacterium*-mediated transient gene expression in *N. benthamiana*

*Agrobacterium* transient gene expression assays were carried out as described by Gao et al. (2011). For co-expression, *A. tumefaciens* GV3101 containing constructs at an  $\text{OD}_{600}$  of 0.4 were mixed together in a 1:1 ratio and infiltrated into the leaves of 4- to 5-week-old *N. benthamiana*. *Agrobacterium* containing the p19-expression plasmid (Voinnet et al., 2003) was always co-infiltrated with *agrobacteria* containing recombinant plasmids used in co-immunoprecipitation and subcellular fractionation assays at a final  $\text{OD}_{600}$  of 0.2.

#### 4.12 | Co-immunoprecipitation assay

The coding sequencing of RIR1-Myc, RIR1-N (amino acids 45–509) -Myc, RIR1-C (amino acids 644–933) -Myc and NUO-HA were cloned into the pXGY vector. The primers are listed in Table S1. All these constructs were transformed into *A. tumefaciens* GV3101 to infiltrate *N. benthamiana*. The total proteins were extracted from tobacco leaves 2 days after infiltration, as described in Qin et al. (2020). The cleared extracts were immunoprecipitated using anti-Myc antibody (ABclonal) and the antibody bound proteins were captured by protein G sepharose (GE Healthcare). The elution resin proteins were detected by western blotting with antibodies against Myc (ABclone) and HA (Cell Signaling Technology).

#### 4.13 | Subcellular fractionation assay and immunoblotting

All subcellular components were isolated as previously described in Zhao et al. (2018) with some modifications. For immunoblotting analysis, cell fractions were extracted from 2-day-postinfiltration tobacco leaves with *Agrobacterium* containing p35S::RIR1-Myc or p35S::NUO-HA plasmids by prechilled TSM buffer (40 mM Tris-HCl

pH 7.5, 20% vol/vol glycerol, 10 mM EDTA, 1 mM dithiothreitol [Sigma] and 1× protease inhibitor cocktail [Roche]) and leaves of *RIR1* overexpression rice plants by prechilled protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM sucrose, 1 mM PMSF, 1 mM EDTA, 1% vol/vol Triton X-100, and 1× protease inhibitor cocktail), respectively. The homogenized solution was centrifuged at 6,000 × *g* at 4 °C for 10 min, then the resulting supernatant was centrifuged at 20,000 × *g* at 4 °C for 60 min to separate the soluble fraction and membrane pellet. The resultant supernatant was referred to as the soluble cytosol fraction and the pellet was referred to as the membrane fraction. The final pellet was resuspended with loading buffer containing the protease inhibitor cocktail. The proteins from cell fractions were immunoprecipitated with anti-Myc, anti-HA, anti-*RIR1*, anti-H<sup>+</sup>-ATPase (Agrisera) antibody. H<sup>+</sup>-ATPase and RuBisCO were used as a plasma membrane marker and a cytoplasmic marker, respectively.

#### 4.14 | In vitro phosphorylation assays

Takara's pCold expression vector was used to express *RIR1-K* and *RIR1-Km* recombinant proteins in *E. coli*. The coding sequence from the native kinase domain (*RIR1K*) was PCR-amplified with primers pCo-*RIR1K-F* and pCo-*RIR1K-R* using *RIR1* cDNA as the template, and then cloned into vector pCold TF using the Trelief SoSoo Cloning Kit (TsingKe). The mutated kinase domain was constructed with primers LM2-F and LM2-R according to the user's manual from the GB Site-Directed Mutagenesis Kit (Genebank Biosciences Inc). His-tagged TF fusion proteins were expressed following the Cold Shock Expression System protocol (Takara) and purified with Ni-NTA Resin (Novagen).

In vitro phosphorylation assays were performed in a reaction mixture containing 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 100 μM ATP, 10 μCi [<sup>32</sup>P]-γATP, 5 μg of myelin basic protein (from bovine brain, MBP; Sigma), and 2 μg of protein with or without 10 mM divalent metal ions (MgCl<sub>2</sub>, CaCl<sub>2</sub>, or MnCl<sub>2</sub>). The reaction mixtures were incubated at 30°C for 3 h and quenched by adding equal volumes of 2× SDS sample buffer (2% SDS, 20% glycerol, 20 mM Tris-HCl pH 6.8, 2 mM EDTA, 160 mM DTT, and 0.1 mg/ml bromophenol blue dye). For the autophosphorylation assay, MBP was omitted from the reaction mixture. The products were resolved on denaturing 12% polyacrylamide gels and visualized using a phosphorimager.

#### 4.15 | ROS measurement

Leaf discs (7 mm) cut from 10-day-old rice were recut three times in parallel to the direction of the leaf vein. The leaves were incubated in sterile distilled water overnight and then dipped into 200 μl of luminol enhancer solution (Promega), to which 50 μg/ml horseradish peroxidase (Jackson Immuno Research) and 1 μM flg22 (Scilight-Peptide) were added, with sterile distilled water as the control. Luminescence was measured by TD-20/20 Luminometry (Turner Designs).

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#### DATA AVAILABILITY STATEMENT

Sequence data from this article can be found in the GenBank/EMBL data libraries at <https://www.ncbi.nlm.nih.gov/genbank/> under accession numbers LOC\_Os12g10740 (*RIR1*), LOC\_Os07g45090 (*NUO*), LOC\_Os03g50885 (*Actin*), LOC\_Os02g41670 (*PAL1*), LOC\_Os11g09010 (*PAD4*), LOC\_Os09g19734 (*ICS1*), LOC\_Os03g12500 (*AOS2*), LOC\_Os01g09800 (*NH1*), LOC\_Os08g39840 (*LOX2*), LOC\_Os10g38060 (*PLD β1*), LOC\_Os07g03710 (*PR1a*), LOC\_Os06g51060 (*PR3*), LOC\_Os12g43380 (*PR5*), LOC\_Os12g36880 (*PR10*), LOC\_Os05g25770 (*WRKY45*). Other data that support the findings of this study are available from the corresponding author on reasonable request.

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## SUPPORTING INFORMATION

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