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The rice OsERF101 transcription factor regulates the NLR Xa1-mediated immunity induced by perception of TAL effectors

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Summary

- Plant nucleotide-binding leucine-rich repeat receptors (NLRs) initiate immune responses by recognizing pathogen effectors. The rice gene Xa1 encodes an NLR with an N-terminal BED domain, and recognizes transcription activator-like (TAL) effectors of Xanthomonas oryzae pv oryzae (Xoo). Our goal here was to elucidate the molecular mechanisms controlling the induction of immunity by Xa1.
- We used yeast two-hybrid assays to screen for host factors that interact with Xa1 and identified the AP2/ERF-type transcription factor OsERF101/OsRAP2.6. Molecular complementation assays were used to confirm the interactions among Xa1, OsERF101 and two TAL effectors. We created OsERF101-overexpressing and knockout mutant lines in rice and identified genes differentially regulated in these lines, many of which are predicted to be involved in the regulation of response to stimulus.
- Xa1 interacts in the nucleus with the TAL effectors and OsERF101 via the BED domain. Unexpectedly, both the overexpression and the knockout lines of OsERF101 displayed Xa1-dependent, enhanced resistance to an incompatible Xoo strain. Different sets of genes were up- or downregulated in the overexpression and knockout lines.
- Our results indicate that OsERF101 regulates the recognition of TAL effectors by Xa1, and functions as a positive regulator of Xa1-mediated immunity. Furthermore, an additional Xa1-mediated immune pathway is negatively regulated by OsERF101.

Introduction

Plants have developed two tiers of immune systems to defend against pathogen infection. The first tier is initiated through recognition of microbe-associated molecular patterns by plasma membrane-localized pattern recognition receptors. This is referred to as pattern-triggered immunity (Dangl et al., 2013). To inhibit pattern-triggered immunity or to improve the nutrient conditions suitable for pathogen proliferation, pathogens deliver a variety of effectors into plant cells (Dou & Zhou, 2012). For the second tier of immune responses, plants have evolved a family of nucleotide-binding (NB) leucine-rich repeat (LRR) receptors (NLRs) that directly or indirectly recognize pathogen effectors (Jones et al., 2016). This is referred to as effector-triggered immunity. Effector-triggered immunity often involves a hypersensitive cell death response (HR). Several NLRs indirectly recognize pathogen effectors by interacting with host factors. These host factors are defined as ‘sensing decoys’ that mimic the targets of pathogen effectors and act as coreceptors with the NLRs (van der Hoorn & Kamoun, 2008; Paulus & van der Hoorn, 2018).

Xanthomonas oryzae pv oryzae (Xoo) causes rice bacterial blight disease, one of the most important rice diseases in the world. Xoo has developed transcription-activator-like (TAL) effectors to facilitate bacterial growth. Transcription activator-like effectors contain a central region of polymorphic repeats (central repeat region, CRR) with each repeat consisting of 34 amino acid residues, several nuclear localization signals (NLSs) and an activation domain at the C terminus. Each of the repeats in the CRR specifies a nucleotide for binding (Boch et al., 2009; Moscow & Bogdanove, 2009). Xoo secretes TAL effectors into rice cells through a type III secretion system, and the TAL effectors then localize to the host nuclei to regulate expression of certain host genes. One of the Xoo TAL effectors, AvrXa7, accelerates expression of the SWEET14 gene, which encodes a plasma membrane-localized sugar transporter (Antony et al., 2010). The enhanced accumulation of SWEET14 protein increases the efflux of sugars from the cytoplasm to the apoplast, and this provides additional nutrients for the pathogen (Naseem et al., 2017). This process is very...
important for bacterial virulence, because defects in the expression of SWEET14 greatly reduce bacterial growth (Oliva et al., 2019).

The bacterial blight disease resistance gene Xa1 was identified originally in the rice cultivar Kogyoku (Yoshimura et al., 1998). It encodes an NLR protein with an N-terminal BED-type zinc finger domain. Xa1 recognizes TAL effectors and induces immune responses including the HR (Ji et al., 2016), but the mechanisms for these processes have not been elucidated. Recently, alleles of Xa1 called Xa2, Xa14, Xa45 and Xa1 have been isolated (Ji et al., 2020; Read et al., 2020; Zhang et al., 2020). Their predicted protein structures indicate that the BED and NB regions are highly conserved, but their C-terminal LRR regions are distinguished by the number of repeats. This suggests that the LRR regions may determine the interactions with TAL effectors (Read et al., 2020).

The immune responses induced when Xa1 and its allelic NLR proteins recognize TAL effectors can be suppressed by interfering TAL (iTAL) effectors, also referred to as truncated TAL (trunc-TAL) effectors (Ji et al., 2016, 2020; Read et al., 2016). iTAL effectors lack the activation domain but retain the NLs. iTAL effectors can be classified into two types (A and B) based on their structures (Ji et al., 2016; Read et al., 2016). However, the molecular mechanisms by which iTAL effectors suppress Xa1-mediated immunity remain to be revealed.

In this study, we found that the BED domain of Xa1 forms a complex with two TAL effectors, AvrXa7 and Xoo1132. To understand the molecular mechanism of Xa1-mediated immunity, we screened for proteins that interact with Xa1, and identified OsERF101/OsRAP2.6, a member of the AP2/ERF-type family of transcription factors. We investigated the interactions between OsERF101 and Xa1 in plants. We also analyzed Xa1-dependent resistance and TAL effector-induced gene expression between OsERF101 and Xa1 in plants. We also analyzed Xa1-dependent resistance and TAL effector-induced gene expression using plants that overexpressed OsERF101 and plants carrying knockout mutations of OsERF101. Furthermore, we found that OsERF101 directly interacts with the TAL effectors. The results of these experiments suggest that OsERF101 is probably involved in both effector recognition and immune activation mediated by Xa1.

Materials and Methods

Plant materials

Rice (Oryza sativa) Japonicum cultivars Kogyoku and Nipponbare were used as the wild-type plants. The x1 and oserf101 mutants were generated using the CRISPR/Cas9 system as described below. The OsERF101-OX plants were generated as described below.

Plant transformation

To construct the plasmids for the CRISPR/Cas9 system, we used the guide RNA cloning vector pU6gRNA and the all-in-one Cas9/gRNA vector p2DgRNA_Cas9ver.2_HPT, which were kindly provided by Dr Masaki Endo (Mikami et al., 2015). The 20 bp sequences from 108 to +127 of Xa1 (5'-GCAACTGTCTGCAAGAGTC-3') and from 206 to +225 of OsERF101 (5'-GTTTTCAGCAGGCATCGG-3') were selected as the target sites of Cas9 by using the CRISPR-P website (http://crib.hzau.edu.cn/cgi-bin/CRISPR). These DNA fragments were cloned into pU6gRNA, and then subcloned into pZDrRNA_Cas9ver.2_HPT (Mikami et al., 2015). Calli generated from rice embryos were transformed using Agrobacterium tumefaciens EHA101 carrying each construct, as described previously (Hiei et al., 1994). To confirm the mutations, the genomic regions containing the Cas9 target sites were amplified by PCR and sequenced as previously described (Mikami et al., 2015). For overexpression, the entire coding region of OsERF101 was amplified with gene-specific primers (Supporting Information Table S1) using cDNAs prepared from cv Kogyoku leaves as the templates, and the PCR product was cloned into the binary vector pGBW2 (Nakagawa et al., 2007). In the resulting construct, the OsERF101 gene was driven by the CaMV 35S promoter. Calli generated from rice embryos were transformed with the construct as described previously (Hiei et al., 1994).

Transient assays using rice protoplasts

Protoplasts were isolated from cultured rice cells by digestion of the cell walls with Cellulase RS (Yakult Pharmaceutical, Tokyo, Japan) and Macerozyme R-10 (Yakult Pharmaceutical) as described previously (Yamaguchi et al., 2013). Aliquots (100 μl) of protoplasts (2.5 × 10^6 cells ml^-1) were transformed with plasmid DNA using the polyethylene glycol (PEG) method (Chen et al., 2010). For subcellular localization analysis and bimolecular fluorescence complementation (BiFC) analysis, transfected protoplasts were observed using a fluorescence microscope, the Axio Imager M2 (Carl Zeiss, Oberkochen, Germany) with the ApoTome2 system (Carl Zeiss). The BiFC analyses were carried out as reported previously (Yamaguchi et al., 2013).

Split NanoLuc luciferase complementation assay

DNA fragments of Xa11–1802, Xa11–325, Xa1312–1012, Xa11008–1802, AvrXa7 and Xoo1132 were transferred into p35S-GW-T7-LgBiT, p35S-GW-T7-SmBiT or p35S-SmBiT-GW-T7 using the Gateway system with LR clonase reactions (Taoka et al., 2021). The Firefly Luciferase gene controlled by the CaMV 35S promoter was used as an internal control. The constructs were transfected into rice protoplasts as described above. After 18 h of incubation at 30°C, the Firefly and NanoLuc luciferases were measured in a TriStar2 LB942 luminometer (Bertold, Bad Wildbad, Germany) using the ONE-Glo Luciferase Assay System (Promega, Madison, WI, USA) and the Nano-Glo Live Cell Assay System (Promega).

Yeast two-hybrid assays

The yeast two-hybrid screening and interaction assays were based on the requirement for histidine for yeast growth, as described previously (Ishikawa et al., 2014).
Protein extraction and immunoblotting

Total proteins were extracted from rice protoplasts in a buffer including 50 mM Tris–HCl pH 7.5, 1 mM EDTA, and a protease inhibitor cocktail (Roche), and analyzed by immunoblotting with α-HA, α-Lg and α-T7.

Pathology assays

Fully expanded rice leaves were inoculated with Xoo T7174 or Xoo T7133 by infiltration of the bacterial suspension (OD600 = 0.25) with a needleless syringe. The bacterial populations in the leaves after infiltration were quantified by quantitative real-time PCR. The DNA levels of the Xoo XopA gene relative to those of the rice ubiquitin gene were measured using genomic DNAs purified from the infected leaves.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from rice leaves using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with RNase-free DNase I (Roche). First-strand cDNA was synthesized from 1μg total RNA using an oligo-dT primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Expression levels were measured by quantitative real-time PCR using the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) in a Step-One Plus Real-Time PCR system (Applied Biosystems). The expression levels were normalized against a ubiquitin reference gene. Three biological replicates were used for each experiment, and two quantitative replicates were performed for each biological replicate.

RNA-sequencing

Total RNA was used to make sequencing libraries using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) according to the manufacturer’s instructions. Subsequently, the libraries were sequenced using an Illumina HiSeq4000 platform to obtain 150 bp paired-end reads, and the RNA-sequencing (RNA-seq) data were deposited in the DNA Databank of Japan (DDBJ) under the Bio-projects accession no. PRJDB13649. FastQC (Lo & Chain, 2014) software was used to filter high-quality reads from the generated sequence reads. The filtered reads were aligned with the rice reference sequence for cv Nipponbare (http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all_dir/all_con/) using HISAT2 (Kim et al., 2015) software, and counted for each gene using FEATURECOUNTS (Liao et al., 2014) software. Differentially expressed genes (DEGs) were detected using a TCC package (Sun et al., 2013). The enrichment analysis of cis regulatory elements was carried out using the Analysis of Motif Enrichment tool of the MEME suite of motif databases (https://meme-suite.org/meme/tools/ame). Gene Ontology (GO) analysis of the DEGs was performed using RiceNetDB (http://bis.zju.edu.cn/ricenetdb/).

Results

Xa1 interacts with TAL effectors in the rice nucleus

To analyze the interactions between Xa1 and TAL effectors, we first used Xa1 protein fusions with green fluorescence protein (GFP) to determine their subcellular localization in transiently expressing rice protoplasts. Green fluorescence protein was fused to the N termini of full-length Xa11-1802 and three different Xa1 regions: Xa11-325, Xa1132-1012 and Xa11008-1802. These three regions contained the BED, NB and LRR domains, respectively (Fig. 1a). GFP-Xa11-325 was localized mainly in the nucleus (Figs 1b,c, S1). GFP-Xa11-1802 was detected in both the nucleus and the cytoplasm, while GFP-Xa132-1012 and GFP-Xa11008-1802 were localized only in the cytoplasm. Thus, it is likely that the BED domain possesses nuclear-localization activity, which is consistent with a recent report (Zhang et al., 2020).

The Xoo strain T7174 (also called MAFF311018) (Ochiai et al., 2005) is incompatible with the rice cultivar Kogyoku, which carries Xa1. Xoo T7174 contains 16 TAL effectors including AvrXa7 and Xoo1132 (Ochiai et al., 2005). AvrXa7 and Xoo1132 have 98.9% sequence identity between their N-terminal regions and 98.4% identity between their C-terminal regions, and they have 22.5 and 12.5 repeats in their central repeat regions, respectively (Figs 1d, S2). AvrXa7 and Xoo1132 were each fused to GFP and transiently expressed in rice protoplasts. Fluorescence from both constructs was detected in the nuclei (Figs 1e, S3). The N-terminal region (1–277 amino acids aa) and C-terminal region (871–1002 aa) of Xoo1132 were also each fused to GFP. Xoo1132371–1002-GFP was localized in the nucleus whereas Xoo11321–277-GFP was detected in both the nucleus and the cytoplasm, as was also observed for the GFP control (Fig. 1e). This result was consistent with the fact that three NLSs exist within the C-terminal region of Xoo1132.

We next performed BiFC experiments to examine the interactions of Xa1 with AvrXa7 and Xoo1132. Full-length Xa11-1802 protein was tagged with the C-terminal domain of Venus (Xoo1132871–1002 aa) and C-terminal region (871–1002 aa) of Xoo1132 were each tagged with the N-terminal domain of Venus (Xoo1132871–1002 aa) and C-terminal region (871–1002 aa) of Xoo1132 were each tagged with the N-terminal domain of Venus (Xoo1132-Vn and AvrXa7-Vn). Although Xa11-1802-Vc was transfected into rice protoplasts, we failed to detect the corresponding protein (Fig. S4a). Therefore, we tagged each of the Xa1 domains Xa11-325, Xa132-1012 and Xa11008-1802 with the N-terminal domain of Venus and used them in the BiFC experiments. Fluorescence was detected in the nuclei when Xa11-325-Vc was coexpressed with Xoo1132-Vn or AvrXa7-Vn, but not when AvrXa7-Vn was coexpressed with Xa132-1012-Vc or Xa11008-1802-Vc (Figs 2a, S4a,b). Since protein levels of Xa132-1012-Vc and Xa11008-1802-Vc in the protoplasts were much lower than that of Xa11-325-Vc (Fig. S4a), we could not exclude the possibility that the NB and LRR domains interact with the TAL effectors. However, these data suggest that at least the BED domain of Xa1 associates with these TAL effectors. We also coexpressed Xa11-325-Vc with constructs containing Vn linked to either the N- or C-terminal regions of Xoo1132 (Xoo1132371–1002, respectively). The BED (Xa11-325) domain interacted with Xoo1132371–1002 but not with...
Xoo1132^{1–277} in the nucleus (Figs 2a, S4b), even though the GFP constructs indicated that Xoo1132^{1–277} and Xoo1132^{871–1002} were both localized in the nucleus (Fig. 1e).

To confirm the interaction between the BED domain and the TAL effectors, we carried out a split NanoLuc luciferase complementation assay (Taoka et al., 2021). Xa1^{1–325} was fused to the Large BiT of NanoLuc Luciferase (LgBiT, 159 aa), while AvrXa7 and Xoo1132 were each fused to the Small BiT (SmBiT, 12 aa). The constructs were transfected into rice protoplasts with the 35S-Firefly Luciferase (F-Luc) construct, and the activities of both luciferases were measured using a microplate luminometer. Consistent with the BiFC experiments, Xa1^{1–325} (the BED domain) interacted with both TAL effectors (Figs 2b,c, S5).

The BiFC and split NanoLuc luciferase complementation experiments suggested direct interaction between the BED domain of Xa1 and the TAL effectors. To further test this interaction, we carried out a split NanoLuc luciferase complementation assay (Taoka et al., 2021). Xa1^{1–325} was fused to the Large BiT of NanoLuc Luciferase (LgBiT, 159 aa), while AvrXa7 and Xoo1132 were each fused to the Small BiT (SmBiT, 12 aa).
possibility, we carried out yeast two-hybrid assays. However, interactions were not detected between the BED domain and either of the TAL effectors (Fig. S6). This result suggested that some host factor(s) might be required for the interaction between the BED domain and the TAL effectors.

OsERF101 interacts with Xa1

To identify host factors involved in the Xa1-mediated immune response, we screened for Xa1 interactors using a yeast two-hybrid assay with the BED domain (Xa11–325) and a rice cDNA library. Initially, we identified 12 candidates (Table S2). Among them, we selected the AP2/ERF-type transcription factor OsERF101/OsRAP2.6 (LOC_Os04g32620). Our selection was based on the predicted subcellular localization of OsERF101 and the reproducibility of its interaction with the BED domain. In addition, we could not confirm the interaction of the other 11 candidates with the BED domain using any methods other than the yeast two-hybrid assay. Yeast two-hybrid experiments indicated that OsERF101 interacted with the BED domain but not with other Xa1 domains (Figs 3a, S7).

When the OsERF101 protein was fused with GFP (OsERF101-GFP) and transiently expressed in rice protoplasts, the protein was localized to the nucleus (Figs 3b, S8a). We used BiFC assays to demonstrate in planta interactions between the BED domain and OsERF101 in the nucleus (Figs 3c, S8b). In addition, we confirmed the interactions between the BED domain and OsERF101 using split NanoLuc luciferase complementation assays (Figs 3d, S8c).

OsERF101 interacts with the TAL effectors

As described above, we did not detect direct interactions between the BED domain and the TAL effectors in yeast two-hybrid assays. This raised the possibility that OsERF101 may function as a link between Xa1 and the TAL effectors. Therefore, we looked for interactions between OsERF101 and the TAL effectors. In split NanoLuc luciferase complementation assays, OsERF101 showed significant interactions with the TAL effectors (Figs 3f, S8), although the interactions were much weaker than those between the BED domain and the TAL effectors. In addition, the yeast two-hybrid experiments demonstrated direct interactions between OsERF101 and the TAL effectors (Figs 3f, S9). We also used BiFC assays to confirm the interactions between OsERF101 and the TAL effectors (Fig. 3g). However, in these experiments, the BED domain interacted with the TAL effectors even in the absence of OsERF101 (Fig. S10). Furthermore, yeast three-hybrid analysis indicated that the BED domain was not able to interact with the TAL effectors in the presence of OsERF101 (Fig. S11). Thus, although our experiments suggest OsERF101 forms a complex with Xa1 and the TAL effectors, it appears that additional components may be required for the interaction between the BED domain and the TAL effectors.

OsERF101 positively regulates bacterial blight resistance

We generated an Xa1 knockout mutant (xa1) in the Kogyou background using the CRISPR/Cas9 system. The mutant carried a two-base deletion located 1022 bp from the start codon...
We then used a needleless syringe-infiltration technique to introduce suspensions of \textit{Xoo} T7174 and \textit{Xoo} T7133 (which is compatible with Kogyoku) into the leaves of wild-type and mutant Kogyoku plants. Wild-type plants developed HR lesions with dark brown edges and weak water soaking when infiltrated with \textit{Xoo} T7174, but showed only water soaking when infiltrated with \textit{Xoo} T7133 (Fig. 4a). By contrast, the \textit{xa1} mutant did not develop HR lesions when infiltrated with \textit{Xoo} T7174. Consistent with those results, the bacterial population of \textit{Xoo} T7174 was much greater in the \textit{xa1} mutant than in the wild-type plants (Fig. 4b). As mentioned in the Introduction section, AvrXa7 induces SWEET14 expression by direct binding to its
promoter (Antony et al., 2010). We observed stronger induction of SWEET14 expression in the xa1 mutant than in the wild-type plants after infiltration with Xoo T7174 (Fig. 4c).

To elucidate the function of OsERF101 in bacterial blight resistance, we transformed wild-type Kogyoku plants with an overexpression construct of OsERF101 driven by the CaMV 35S promoter (Fig. S13). When these plants were infiltrated with the Xoo T7174 suspension, they exhibited a stronger HR than the wild-type plants, as indicated by the development of HR lesions without water soaking (Fig. 4a). Consistent with the stronger HR, both bacterial growth and SWEET14 expression were reduced in the infiltrated OsERF101-OX plants when compared with nontransformed plants (Fig. 4b,c). These results indicate that OsERF101 plays a positive role in resistance to bacterial blight.

Knockout of OsERF101 results in enhanced resistance

To analyze the involvement of OsERF101 in Xa1-mediated immunity, we used the CRISPR/Cas9 system to generate two knockout mutant lines of OsERF101 in the Kogyoku background. Both oserf101-1 and oserf101-2 carried frame-shift mutations located c. 220 bp from the start codon (Fig. S12b). When either of these lines was infiltrated with Xoo T7174, it exhibited light brown lesions that had a different appearance from the typical Xa1-induced HR lesion (Fig. 4a). This result was unexpected because OsERF101 was predicted to function as a positive regulator of rice immunity. However, bacterial growth and SWEET14 expression were strongly suppressed in the oserf101 mutants after infiltration with Xoo T7174 (Fig. 4b,c), as we also observed in the OsERF101-OX plants. Thus, both the overexpression of OsERF101 and the knockout of OsERF101 induced strong resistance to rice bacterial blight.

The enhanced resistance of OsERF101-OX and oserf101 plants depends upon Xa1

The Xoo strain T7133 is compatible with Kogyoku (Ogawa et al., 1978) and produces disease lesions with water soaking when infiltrated into the leaves of Kogyoku (Fig. 4a). We determined the genome sequence of Xoo T7133 and found that it contains a type-A iTAL effector gene (Yoshikisa et al., 2021). Thus far, all Xoo strains containing type-A iTAL effectors have been observed to inhibit Xa1-mediated immunity in rice (Ji et al., 2020). Therefore, we expect that the iTAL effector of Xoo T7133 would also inhibit Xa1-mediated resistance.

We infiltrated a suspension of Xoo T7133 into the leaves of the OsERF101-OX plants. The OsERF101-OX lesions did not exhibit HR lesions as they did with Xoo T7174, but instead displayed water-soaked disease lesions (Fig. 4a). Consistent with this result, the bacterial populations of Xoo T7133 were increased in the OsERF101-OX plants, as they were in the wild-type plants and the xa1 mutant (Fig. 4d). In addition, expression levels of SWEET14 in the Xoo T7133-infiltrated OsERF101-OX plants were similar to those in the wild type and the xa1 mutant (Fig. 4e). We also infiltrated a suspension of Xoo T7133 into the leaves of the oserf101 knockout mutant lines. The atypical HR lesions induced by infection with Xoo T7174 were not observed after infection with Xoo T7133 (Fig. 4a). As observed in the OsERF101-OX plants, the infiltrated oserf101 leaves developed water-soaked disease lesions (Fig. 4a), along with wild-type levels of bacterial growth and SWEET14 expression (Fig. 4d,e). Because the iTAL effector of Xoo T7133 probably suppresses the immune responses induced via the recognition of TAL effectors by Xa1, these results suggest that the enhanced resistance of the OsERF101-OX and oserf101 plants may be Xa1-dependent.

To confirm that the enhanced resistance induced by both the overexpression and knockout of OsERF101 in the Kogyoku background is dependent on Xa1, we next conducted transient assays to analyze Xa1-mediated cell death. Kogyoku protoplasts were transfected with a dexamethasone (DEX)-inducible AvrXa7 construct (pTA7002::AvrXa7) along with a p35S-NanoLuc luciferase construct, and cell death levels were estimated by measuring the luciferase activity after mock or DEX treatments. DEX treatment induced cell death in the wild-type Kogyoku protoplasts (Fig. S14a). The AvrXa7-induced cell death in Kogyoku protoplasts was reduced by silencing of Xa1. Silencing of Xa1 also suppressed cell death in the OsERF101-OX and oserf101 protoplasts (Fig. S14b,c). These results indicate that the hypersensitive response induced in the OsERF101-OX and oserf101 cells is mainly dependent on Xa1, although weak contributions of other factors in addition to Xa1 cannot be excluded.

To further confirm that the enhanced resistance induced by the overexpression and knockout of OsERF101 is dependent on Xa1, we generated transgenic plants overexpressing OsERF101 and created knockout mutants of OsERF101 in the rice cultivar Nipponbare background (Fig. S12c). Nipponbare does not possess the Xa1 gene. Similar to wild-type Nipponbare leaves, OsERF101-OX and oserf101 Nipponbare leaves developed water-soaked disease lesions when infiltrated with Xoo T7174 (Fig. 5a). In addition, we analyzed bacterial growth and expression of SWEET14 in the Nipponbare lines after infiltration with Xoo T7174 (Fig. 5b,c). The results indicated that neither the overexpression nor the knockout of OsERF101 caused enhanced resistance to Xoo T7174 in the Nipponbare background. Thus, it is likely that the enhanced resistance induced by both the overexpression and knockout of OsERF101 in the Kogyoku background is dependent on Xa1.

As describe above, neither the overexpression nor the knockout of OsERF101 in the Kogyoku background altered its response to the compatible pathogen Xoo T7133. Previously, OsERF101/OsRAP2.6 was reported to positively regulate resistance to a compatible blast fungus. Therefore, we analyzed blast resistance using the OsERF101-OX and oserf101 plants in the Nipponbare background. Unlike the previous report, the OsERF101-OX plants exhibited enhanced susceptibility to a compatible isolate (A92-06-2) of blast fungus (Fig. S15), whereas the oserf101 mutation did not affect the resistance. This inconsistency with the previous report might be due to the differences in rice varieties and the fungal isolates. Thus, it seems that OsERF101 may be involved in the general immune responses against compatible isolates of blast fungus. On the other hand, it is likely that...
Fig. 4 OsERF101-OX and oserf101 enhance bacterial blight resistance in the cv Kogyoku background. (a) Hypersensitive responses of wild-type (WT), the OsERF101-OX and oserf101 plants in the Kogyoku background. Suspensions of *Xanthomonas oryzae pv oryzae* (Xoo) T7174 or Xoo T7133 were injected into the leaves of 3-wk-old seedlings. Photos were taken at 4 d after inoculation (DAI). (b) The bacterial populations of Xoo T7174 in the OsERF101-OX and oserf101 plants were analyzed by quantitative real-time PCR. The data indicate DNA levels of the *X. oryzae XopA* gene relative to those of the rice *ubiquitin* gene. Values are means ± SE. Different letters above the 4 DAI data points indicate significant differences (two-sided Welch’s *t*-test, *P* < 0.01). (c) Expression of SWEET14 in the OsERF101-OX and oserf101 plants after infection with Xoo T7174. Transcript levels were measured by quantitative real-time PCR. Values are means ± SE. Different letters above the 4 DAI data points indicate significant differences (two-sided Welch’s *t*-test, *P* < 0.01). (d) The bacterial populations of Xoo T7133 in the OsERF101-OX and oserf101 plants in the Kogyoku background were analyzed by quantitative real-time PCR. The data indicate DNA levels of the *X. oryzae XopA* gene relative to those of the rice *ubiquitin* gene. Values are means ± SE. Different letters above the 4 DAI data points indicate significant differences (two-sided Welch’s *t*-test, *P* < 0.05). (e) Expression of SWEET14 in the OsERF101-OX and oserf101 plants after infection with Xoo T7133. Transcript levels were measured by quantitative real-time PCR. Values are means ± SE. Different letters above the 4 DAI data points indicate significant differences (two-sided Student’s *t*-test, *P* < 0.05).
OsERF101 contributes specifically to Xa1-mediated immunity in bacterial blight resistance.

To test whether OsERF101 is involved in other incompatible interactions between rice and Xoo, we analyzed cell death responses induced by the bacterial blight resistance gene Xa10. It has been reported that Xa10 expression is directly activated by the TAL effector AvrXa10, which confers race-specific resistance to rice bacterial blight (Tian et al., 2014). Transient expression of Xa10 induced similar levels of cell death in wild-type, OsERF101-OX and oserf101 rice protoplasts (Fig. S16). These results suggest that OsERF101 does not contribute to Xa10-induced immunity.

Overexpression and knockout of OsERF101 induce different types of immune responses

We observed that the OsERF101-OX lines and oserf101 knockout lines in the Kogyoku background displayed different types of HR lesions after infiltration with Xoo T7174 (Fig. 4a). This suggested that OsERF101-OX and oserf101 may have different effects on the expression of downstream genes. Therefore, we carried out RNA-seq analyses using mRNAs purified from wild-type, OsERF101-OX and oserf101 leaves at 2 d after mock treatment or infiltration with Xoo T7174. We identified 5390 DEGs (q-value < 0.01, fold-change > 4) whose expression levels were up- or downregulated when compared with their expression after mock treatment (Fig. 6a; Table S3). There were larger numbers of DEGs in wild-type leaves than in OsERF101-OX or oserf101 leaves. Because bacterial growth in the OsERF101-OX and oserf101 leaves was much lower than that in wild-type leaves (Fig. 4b,c), it seems that the immune responses may be induced at earlier time points in the overexpression and knockout lines. Venn diagrams also indicated that the overexpression of OsERF101 affects the expression of a largely different set of downstream genes than those affected by the knockout mutation of OsERF101 (Fig. 6a). In addition, we carried out GO enrichment analysis of the DEGs shown in Table S3. The results indicate that many DEGs are involved in regulation of response to stimulus (Table S4).

We performed clustering analysis using the 1630 DEGs with transcripts per million (TPM) > 50 (Fig. 6b; Table S4). The experiments revealed four clusters specific to either oserf101 or OsERF101-OX. Clusters #1 and #2 were downregulated specifically in oserf101 and OsERF101-OX, respectively. Clusters #3 and #4 were upregulated specifically in OsERF101-OX and oserf101, respectively. These clusters contained many genes that are predicted to be involved in immune responses (Table S5). Thus, it is likely that the mechanism by which the knockout of OsERF101 enhances Xa1-mediated immunity is different from the mechanism by which OsERF101 overexpression enhances immunity. Our results suggest two regulatory pathways, both mediated by Xa1. In one pathway, OsERF101 functions as a positive immune regulator, whereas the other pathway is negatively regulated by OsERF101.

To further investigate the different transcriptional regulation pathways between the OsERF101-OX and oserf101 plants, we carried out enrichment analyses of cis regulatory elements in the promoter sequences of the DEGs discussed above. We found that the E-box ‘CANNTG’, the cis-element recognized by basic Helix-Loop-Helix (bHLH)-type transcription factors, is enriched in the 360 DEGs specifically downregulated in the OsERF101-

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OX plants (Figs 6a, S17), whereas no enrichment was observed in the 208 DEGs specifically upregulated in the OsERF101-OX plants. On the other hand, the cis-element for Myb-type transcription factors is enriched in the 114 DEGs specifically downregulated in the oserf101 plants (Figs 6a, S18). As in the OsERF101-OX plants, no enrichment was detected in the 105 DEGs specifically upregulated in the oserf101 plant. Thus, it is likely that overexpression of OsERF101 enhances resistance through bHLH-type transcription factors, while the knockout of OsERF101 enhances resistance through Myb-type transcription factors.

**Discussion**

Plants contain large numbers of NLR proteins. Some NLRs possess integrated decoy domains that are targeted by pathogen effectors. These integrated decoy domains mimic other effector targets whose binding to the effectors leads to disease development. Although BED was predicted to be an integrated decoy domain (Zuluaga et al., 2017), direct evidence for this has not yet been obtained. Here, we showed that Xa1 forms complexes with the Xoo TAL effectors AvrXa7 and Xoo1132 via the BED domain, although direct interaction between the BED domain...
and the TAL effectors was not detected. We also found that the BED domain and the TAL effectors interact with the transcription factor OsERF101. Overexpression of OsERF101 enhanced Xa1-mediated disease resistance, suggesting that OsERF101 plays a pivotal role in Xa1-mediated recognition of the TAL effectors and immune activation. Taken together, our results suggested that OsERF101 is a positive regulator in Xa1-mediated immunity.

On the other hand, we found that the *oserf101* knockout mutants also showed enhanced resistance to *Xoo* T7174. Interestingly, the HR lesions of the Kogyoku *oserf101* knockout mutants exhibited quite different characteristics from those of wild-type and *OsERF101-OX* plants. Furthermore, the *OsERF101-OX* plants and the *oserf101* mutants influenced the transcription of largely different sets of downstream genes. Enrichment analyses of *cis* regulatory elements suggested that these different sets of downstream genes are regulated by different types of transcription factors: bHLH-type transcription factors in *OsERF101-OX* plants and Myb-type transcription factors in *oserf101* plants. This phenomenon suggests an additional Xa1-mediated immune pathway that is negatively regulated by OsERF101. Based on these data, we propose a model for Xa1-OsERF101-mediated immune signaling by hypothesizing the existence of an ‘X factor’ involved in Xa1-mediated immunity (Fig. 6c). In this model, both OsERF101 and the X factor positively regulate Xa1-mediated immunity, but OsERF101 has the ability to inhibit activity of the X factor. Overexpression of *OsERF101* induces an immunity pathway mediated by bHLH transcription factors, but may suppress the X factor. Knockout of *OsERF101* results in enhancement of the X factor-mediated immune response through Myb transcription factors. This model is consistent with two observations: the enhanced resistance to *Xoo* T7174, induced by either the overexpression of *OsERF101* or the knockout of *OsERF101* in the Kogyoku background, is not observed with *Xoo* T7133, which carries an iTAL effector; and neither *OsERF101-OX* nor the *oserf101* mutation enhances *Xoo* T7174 resistance in the Nipponbare background, which does not carry Xa1. Note here that *Xoo* T7174 also contains an iTAL effector named Tal3b, but Tal3b is not functional in *Xoo* T7174 for unknown reasons (Ji et al., 2016).

Rice contains 163 AP2/ERF-type transcription factors (Sharoni et al., 2011). The AP2/ERF transcription factors positively or negatively regulate abiotic and biotic stress and hormone responses (Xie et al., 2019). In fact, OsERF101/OrRAP2.6 has been reported to participate in resistance to rice blast (Wamaita et al., 2012), leaf senescence (Lim et al., 2020) and drought stress (Jin et al., 2018). Recently, OsERF101 was reported to regulate the transcription of OsMYC2, which encodes a bHLH transcription factor, by directly binding its promoter (Lim et al., 2020). In addition, overexpression of OsMYC2 is known to enhance resistance to bacterial blight (Uji et al., 2016). These observations suggest that the OsERF101-OsMYC2 module may control *Xoo* resistance. In this study, we found that the cis-elements of the bHLH transcription factors are enriched in the DEGs downregulated in the *OsERF101-OX* plants. Therefore, the bHLH transcription factors including OsMYC2, which are positively regulated by OsERF101, may suppress expression of the corresponding DEGs, although it is possible that OsERF101 may inhibit the bHLH transcription factors that positively regulate expression of the DEGs (Fig. 6c). In a similar way, it appears that the Myb transcription factors may function downstream of the X factor. Further analysis will be required to elucidate how bHLH and Myb transcription factors regulate the Xa1-mediated immunity.

The Xa1 alleles *Xa2*, *Xa14*, *Xa45* and *Xa1* also encode BED-NLR proteins. The BED and NB domains are highly homologous among these Xa1 allelic members. However, they are differentiated by the numbers of repeats in their C-terminal LRR regions (Ji et al., 2020; Read et al., 2020; Zhang et al., 2020), and they confer different resistance spectra to races of *Xoo*. The differences in the LRR regions suggest that the LRR domains may be the determinants of race specificity to *Xoo*. Since the immune responses mediated by these allelic members are suppressed by iTAL effectors (Ji et al., 2020), it is likely that they all recognize the TAL effectors. However, here we did not detect interactions between the LRR domain of Xa1 and the two TAL effectors. Instead, our BiFC and split NanoLuc luciferase complementation assays suggested the formation of a complex involving a TAL effector, OsERF101, and the BED domain of Xa1. Therefore, it is possible that the differences among the LRR regions of the Xa1 allelic members may affect either the formation of the tertiary complex or the LRR domain-mediated recognition of the tertiary complex.

The yeast two-hybrid experiments indicated that OsERF101 directly interacts with Xa1 and the TAL effectors. In addition, OsERF101 regulates Xa1-dependent immunity, and we speculate that the putative X factor also regulates Xa1-dependent immunity. Host factors that are targeted by pathogen effectors and act as coreceptors with NLRs are referred to as sensing decoys (Paulus & van der Hoorn, 2018). Therefore, it is possible that OsERF101 and the X factor may be sensing decoys targeted by the TAL effectors, and that they function as coreceptors with Xa1. Since sensing decoys generally promote effector recognition in the presence of their cognate NLR proteins (Paulus & van der Hoorn, 2018), this scenario is consistent with the fact that the overexpression and knockout mutation of OsERF101 enhanced Xa1-dependent immunity in the Kogyoku background, but not in the Nipponbare background. However, since our data suggest that OsERF101 activates immunity through bHLH transcription factors, it appears that OsERF101 also contributes to the activation of Xa1-mediated immune signaling.

Consistent with recent reports (Read et al., 2020; Xu et al., 2021), we found that Xa1 is localized in the nucleus. Our data indicated that the BED domain can confer nuclear localization. Thus, it is likely that Xa1 recognizes the TAL effectors in the nucleus. In fact, the inhibition of Xa1-mediated immunity by the iTAL effectors requires the nuclear localization of the iTAL effectors (Ji et al., 2016). These data suggest strongly that Xa1 activates immune responses within the nucleus. Recent investigations using coimmunoprecipitation indicated that the Xa1 allelic member Xo1 interacts with the iTAL effector Tal2h (Read et al., 2020), although it is not yet known whether the interaction...
is direct or indirect. Although the molecular mechanisms of how the iTAL effectors inhibit Xa1-mediated immunity are not yet understood, it is possible that the iTAL effectors may suppress Xa1 through OsERF101 or the putative X factor.

It has been reported that the BED domains form dimers (Zhang et al., 2020). This suggests that oligomerization to form resistosomes may occur in BED NLRs, as is the case with other NLR proteins (Wang et al., 2019; Ma et al., 2020; Martin et al., 2020). If interactions among the BED domain, OsERF101 and the TAL effectors alter the tertiary structure of the Xa1 protein, it is possible that this structural change may facilitate oligomerization through the BED domains. Recent structural studies of resistosomes using cryoelectron microscopy are beginning to reveal how NLRs activate immunity (Wang et al., 2019; Ma et al., 2020; Martin et al., 2020). Some NLRs with the N-terminal Toll-interleukin-1 receptor domain induce cell death through their nicotinamide adenine dinucleotide hydrolase activity and/or their cyclic nucleotide monophosphate synthetase activity (Horsefeld et al., 2019; Wan et al., 2019; Yu et al., 2022). On the other hand, several NLRs with N-terminal coiled coil domains function as plasma membrane-localized calcium-permeable channels (Bi et al., 2021; Jacob et al., 2021). However, the molecular mechanisms by which nuclear-localized NLRs activate immunity in plants are still unknown. The present report on the interaction between Xa1 and OsERF101 in initiation of the immune response provides new insights into NLR-mediated immunity.

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Author contributions

AY, SY and TK designed the research. AY, SY, SS, SM and KY performed the experiment. MS and AM analyzed RNA-seq data. AY, SY and TK wrote the manuscript. AY and SY contributed equally to this work as first coauthors.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Western blot analysis of green fluorescence protein-fused Xa1 and its truncated proteins for the experiment shown in Fig. 1(b).

**Fig. S2** Alignment of the amino acid sequences in the N- and C-terminal regions of Xoo1132 and AvrXa7.

**Fig. S3** Western blot analysis of green fluorescence protein–fused AvrXa7, Xoo1132, Xoo1132*Avr* and Xoo1132*Avr* for the experiments shown in Fig. 1(c).

**Fig. S4** Western blot analysis of the Xa1 and transcription activator–like effector proteins for the bioluminescent fluorescence complementation experiments shown in Fig. 2(a).

**Fig. S5** Western blot analysis of LgBiT–fused Xa1*–325* protein and SmBiT–fused transcription activator–like effectors in split NanoLuc luciferase assays for the experiments shown in Fig. 2(b,c).
**Fig. S6** Interaction of Xa1$^{1−325}$ with Xoo1132 and AvrXa7.

**Fig. S7** Western blot analysis of the Xa1-truncated proteins and the OsERF101 protein for the two-hybrid assay shown in Fig. 3 (a).

**Fig. S8** Western blot analysis of the proteins expressed in rice protoplasts for the experiments shown in Fig. 3(b–e).

**Fig. S9** Western blot analysis of OsERF101, Xa1$^{1−325}$ and the transcription activator-like effectors for the two-hybrid assay shown in Fig. 3(f).

**Fig. S10** The BED domain of Xa1 interacts with the transcription activator-like effectors in OsERF101-OX and oserf101 cells.

**Fig. S11** Yeast three-hybrid assay showing the lack of interaction between the BED domain and the transcription activator-like effectors in the presence of OsERF101.

**Fig. S12** The mutations produced in this study.

**Fig. S13** Transcript levels of OsERF101 in leaves of the transgenic plants overexpressing OsERF101.

**Fig. S14** Cell death assays using a DEX-inducible AvrXa7 system.

**Fig. S15** Blast resistance of OsERF101-OX and oserf101 plants.

**Fig. S16** Cell death responses induced by transient expression of Xa10.

**Fig. S17** *Cis* regulatory elements enriched in genes downregulated in OsERF101-OX plants.

**Fig. S18** *Cis* regulatory elements enriched in genes downregulated in oserf101 plants.

**Table S1** Primers used in this study.

**Table S2** Candidate Xa1 interactors.

**Table S3** Differentially expressed gene list and annotations.

**Table S4** Gene Ontology enrichment analysis of the differentially expressed genes.

**Table S5** List of genes identified by clustering analysis.

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