TALE-carrying bacterial pathogens trap host nuclear import receptors for facilitation of infection of rice

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SUMMARY
Many plant-pathogenic Xanthomonas rely on the secretion of virulence transcription activator-like effector (TALE) proteins into plant cells to activate plant susceptibility genes to cause disease. The process is dependent on the binding of TALEs to specific elements of host target gene promoters in the plant nucleus. However, it is unclear how TALEs, after injection into host cells, are transferred from the plant cytoplasm into the plant nucleus, which is the key step of successful pathogen infection. Here, we show that the host plant cytoplasm/nuclear shuttle proteins OsImp1a and OsImp1b are key components for infection by the TALE-carrying bacterial pathogens Xanthomonas oryzae pv. oryzae (Xoo) and Xanthomonas oryzae pv. oryzicola (Xoc), the causal agents of bacterial leaf blight and bacterial leaf streak, respectively, in rice. Direct interaction between the second nuclear localization signal of TALEs of Xoo or Xoc and OsImp1a or OsImp1b is required for the transportation of TALEs into the nucleus. Conversely, suppression of the expression of OsImp1a and OsImp1b genes attenuates the shuttling of TALEs from the cytoplasm into the nucleus and the induction of susceptibility genes, thus improving the broad-spectrum disease resistance of rice to Xoo and Xoc. These results provide an applicable strategy for the improvement of resistance to TALE-carrying pathogens in rice by moderate suppression of the expression of plant nuclear import receptor proteins.

Keywords: nuclear import receptor, nuclear localization signals, rice, transcription activator-like effector, Xanthomonas

INTRODUCTION
Xanthomonas, which is a large group of Gram-negative bacterial plant pathogens, consists of almost 30 species and causes diseases on at least 124 monocotyledonous and 268 dicotyledonous plants (Ryan et al., 2011). Many Xanthomonas bacteria secrete transcription activator-like effectors (TALEs), the major virulence and determinant factors in host–pathogen interactions, into plant cells, where they act as transcriptional activators to reprogram the host plant transcriptome. TALEs function as eukaryotic transcription factors, which activate the transcription of host susceptibility genes to cause susceptibility of plants or induce the expression of host resistance genes to cause resistance of plants, and rely on their injection into the plant nucleus and their binding to the effector binding element (EBE) of host target gene promoters (Cox et al., 2017; Zhang et al., 2015). TALEs are highly conserved, with homologues across Xanthomonas species sharing greater than 90% amino acid identity and almost identical structural features. In general, TALEs typically consist of the following: an N-terminal type III secretion signal that guides the translocation of TALEs from the bacterium into the host plant cytoplasm through the type III secretion system (T3SS); a central repeat region (RR), which is the main hallmark of TALEs, that specifically binds to EBE of the host gene promoter; a transcription factor binding (TFB) motif that hijacks the host plant basal transcription factor IIA γ subunit to fulfil the binding of TALEs to EBE; three short nuclear localization signals (NLSs) that guide the translocation of TALEs from the plant cytoplasm into the plant nucleus; and a highly conserved acidic activation domain (AD) that allows TALEs to activate gene transcription in plant cells (Doyle et al., 2013; Huang et al., 2017; Yuan et al., 2016; Zhang et al., 2015).

Xanthomonas oryzae pv. oryzicola (Xoc) causes bacterial leaf blight of rice and Xanthomonas oryzae pv. oryzae (Xoo) causes bacterial leaf streak of rice, both of which are devastating bacterial diseases. Massive efforts have been undertaken to decipher Xoo and Xoc TALE biology. Numerous susceptibility genes in rice targeted by TALEs have been forecasted and validated. TALEs pthXo1, pthXo2 and pthXo3/AvrXa7/Tal5/TalC/TalF of Xoo induce the expression of Xa13/Os8N3/SWEET11, Xa25/Os12N3/SWEET13 and Xa41/Os11N3/SWEET14, respectively, which are three members of the rice MtN3/saliva/SWEET family and function as sucrose transporters (Antony et al., 2010; Blanvillain-Baufumé et al., 2017; Chu et al., 2006; Hutin et al., 2015; Liu et al., 2011; Streubel et al., 2013; Tran et al., 2018; Yang et al., 2006; Zhou et al., 2015). TALEs pthXo6, TalB and pthXo7 of Xoo up-regulate the transcription factors
OsTFX1, OsERF123 and OsTFL1-Ay1, respectively (Sugio et al., 2007; Tran et al., 2018). TALEs Tal9a of Xoo and Tal1c of Xoc trigger the induction of OsHen1, which encodes a protein with a predicted methyltransferase domain involved in micro-RNA (miRNA) maturation (Moscou and Bogdanove, 2009). TALE Tal2g of Xoc activates OsSULTR3;6, which encodes a sulfate transporter (Cernadas et al., 2014). All of these susceptibility genes can benefit Xoo or Xoc multiplication and disease development after activation by TALEs, although they encode different types of protein. Xoo and Xoc contain 8–26 and 19–29 TALEs, respectively, based on genome sequence (Booher et al., 2007). To improve plant resistance to the TALE-carrying bacterial pathogen Xanthomonas, TALE-carrying a specific group of proteins (Goldfarb et al., 2004; Peters, 2006), the best characterized of which is the import of proteins containing a classical NLS that consists of either a short stretch of three to five basic amino acids or two basic domains separated by a spacer. The NLS-containing proteins are recognized and bound in the cytoplasm by the NLS receptor, and then transported into the nucleus. In rice, importin proteins OsImpα1a and OsImpα1b, which function as nucleocytoplasmic transporters, selectively bind and transport proteins containing T-NLS (a monopartite-type NLS with typical amino acid residues CTPKKKKRKV) and O2-NLS (a bipartite-type NLS with typical amino acid residues MPTEERVKKRKESNRESARRYRKAALHKC), but not R-NLS (a yeast Matα2-type NLS with typical amino acid residues CYMISEALKAIGKR) (Chang et al., 2012, 2014; Goldfarb et al., 2004; Jiang et al., 1998, 2001). Here, we reveal that rice OsImpα1a and OsImpα1b coordinately transfer the TALEs of Xoo and Xoc from the plant cytoplasm into the nucleus by selectively binding the bacterial pathogen-derived NLS of TALEs, which features a short stretch of five amino acids rich in arginine and lysine residues (RKRRS). We further demonstrate that OsImpα1a and OsImpα1b (cytoplasm/nucleus shuttle proteins)-mediated transportation of TALEs is vital for Xoo- and Xoc-triggered induction of targeting of susceptibility genes. Our results suggest that modification of the host nuclear import receptor genes OsImpα1a and OsImpα1b of rice by moderate suppression may provide a universally applicable strategy to improve plant resistance to the TALE-carrying bacterial pathogens Xoo and Xoc.

**RESULTS**

**Rice OsImpα1a interacts with Xoo pthXo1**

Like other Xoo TALEs, pthXo1 also typically contains an amino-terminal translocation signal (TS), a central RR, a newly identified TFB motif, three NLSs and a carboxyl-terminal transcription AD. Our previous studies have demonstrated that the complete pthXo1 possesses auto-activation activity because of the presence of the TS or AD domain; the truncated pthXo1 containing RR-TFB-NLS does not show auto-activation transcription activity in yeast (Yuan et al., 2016). To screen for pthXo1-interacting host rice proteins, the RR-TFB-NLS fragment of pthXo1 was used as a bait to trap proteins putatively interacting with pthXo1 by yeast two-hybrid assay. The prey cDNA library was prepared from mRNA isolated from leaves of rice variety IR24 after inoculation with Xoo strain PX099. Several candidate pthXo1-interacting proteins were identified from approximately 5 x 10<sup>5</sup> independent colonies screened. Among the putative interacting proteins, the cDNA for the rice importin α1 gene (hereafter designated as OsImpα1a) showed the strongest interaction with the RR-TFB-NLS fragment of pthXo1. Rice OsImpα1a encodes a protein of 526 amino acids and has high amino acid sequence similarity with its homologues in rice, such as OsImpα1b and OsImpα2 (Jiang et al., 1998, 2001). A phylogenetic tree of plant importin α proteins, obtained from Xanthomonas-invaded host plants, showed that OsImpα1a grouped with Arabidopsis AtImpα1 and AtImpα2, pepper Clnα2 and citrus Clnα2 (Fig. S1, see Supporting Information); Clnα2 has been shown to interact with Xanthomonas campestris pv. vesicatoria (Xcv) TALE AvrBs3 in yeast (Szukuk et al., 2001).

**pthXo1 interacts with OsImpα1a and OsImpα1b through NLS2**

To narrow down which domain of pthXo1 interacts with OsImpα1a, we generated a series of pthXo1 constructs in which truncated coding sequences of pthXo1 were translationally fused with the DNA-binding domain of GAL4. These constructs included the entire RR to NLSs (RR-TFB-NLS), the TFB motif with NLSs (TFB-NLS), TFB, the three NLSs (NLS1/2/3), the first and second NLSs (NLS1/2) and the three different NLSs (NLS1, NLS2, NLS3) (Fig. 1A). The resulting constructs were co-transformed into yeast cells with the complete coding sequence of OsImpα1a fused with the DNA AD of GAL4, and empty vectors as the negative controls. The second NLS (NLS2) was the only domain to interact with OsImpα1a in the yeast two-hybrid assay (Fig. 1B). In contrast, RR, TFB, NLS1 and NLS3 did not exhibit interaction with OsImpα1a in yeast (Fig. S2A, see Supporting Information). Further, we found that NLS2 of pthXo1 also interacted with OsImpα1b, but not with...
OsImpα2, in yeast two-hybrid assay (Fig. S2A). These results demonstrate that the second NLS of pthXo1 is necessary for the interaction with the two rice nuclear import receptor proteins OsImpα1a and OsImpα1b.

**pthXo1 interacts with OsImpα1a and OsImpα1b in vivo**

To validate the yeast two-hybrid results, the interaction between pthXo1 and OsImpα1a or OsImpα1b was verified by in vivo assay. We first performed a firefly split-luciferase complementation assay in Nicotiana benthamiana leaves to investigate the interaction. During this assay, pthXo1 was fused with the N-terminal (nLUC) of firefly luciferase; OsImpα1a, OsImpα1b and OsImpα2 were fused with the C-terminal (cLUC) of firefly luciferase. We transiently co-expressed pthXo1-nLUC and OsImpα1a-cLUC, pthXo1-nLUC and OsImpα1b-cLUC, pthXo1-nLUC and OsImpα2-cLUC, as well as pthXo1-nLUC and cLUC as negative controls, in N. benthamiana leaves. A strong fluorescence signal was observed in leaves that co-expressed pthXo1-nLUC and OsImpα1a-cLUC, or pthXo1-nLUC and OsImpα1b-cLUC, but not in pthXo1-nLUC and OsImpα2-cLUC, or negative controls (Fig. 1C), suggesting that pthXo1 interacted with OsImpα1a and OsImpα1b, but not OsImpα2, in planta.

We then performed co-immunoprecipitation (Co-IP) assays using pthXo1-myc and OsImpα1a-FLAG, OsImpα1b-FLAG or OsImpα2-FLAG co-expressed transiently in N. benthamiana leaves. The OsImpα1a-FLAG, OsImpα1b-FLAG and OsImpα2-FLAG proteins were immunoprecipitated using anti-FLAG-conjugated agarose. Immunoblots were washed and probed with anti-myc antibodies. The pthXo1-myc protein was pulled down by OsImpα1a-FLAG and OsImpα1b-FLAG. In contrast, OsImpα2-FLAG could not pull down pthXo1-myc protein in the same conditions (Fig. 1D). Taken together, these results demonstrate that Xoo TALE pthXo1, via the second NLS, physically associates with host rice OsImpα1a and OsImpα1b.

**NLS2 of TALE is essential for Xoo virulence**

We confirmed that Xoo TALE pthXo1 interacts with OsImpα1a and OsImpα1b in vitro and in vivo. We narrowed down the core
Fig. 2  Effect of transcription activator-like effector (TALE) pthXo1 NLS2 on the virulence of Xanthomonas oryzae pv. oryzae (Xoo) strains. (A) Interaction between point-mutated NLS2 of pthXo1 and OsImpα1a analysed by yeast two-hybrid assay. The interactions were assessed by the growth of yeast cells on synthetic defined premixed (SD) medium lacking (–) leucine (L), tryptophan (W), histidine (H) and adenine (A). (B) Detection of interactions between different mutated pthXo1 and OsImpα1a in planta by co-immunoprecipitation (Co-IP). The protein–protein interaction assays were performed in Nicotiana benthamiana leaf cells. Protein before (Input) and after immunoprecipitation (IP) were detected with anti-myc and anti-FLAG antibodies. (C) Expression of pthXo1 targeting susceptibility gene Xa13 after infection of different strains. Asterisks indicate a significant difference between non-infected plants and Xoo-infected plants at **P < 0.01. hpi, hours post-inoculation. (D) Virulence of strain PH and its derivatives carrying pthXo1 or NLS2-mutated pthXo1 in rice. PH is an engineered TALE-free strain with the genetic background of strain PXO99A, which carries pthXo1.
interaction region of pthXo1 to NLS2. When aligning the NLS2 of TALEs from genome sequenced Xoo and Xoc strains, we found that all of the TALEs of different Xoo and Xoc strains exclusively have the same amino acid residues of their NLS2 (RKKR). To assess which amino acid residues of RKKR in NLS2 are essential for TALE function or Xoo virulence, we first produced pthXo1 derivatives with the five conserved amino acid residues of their NLS2 (RKRSR). To investigate the intracellular localization of OsImp1a and OsImp1b, we transiently expressed OsImp1a-FLAG and OsImp1b-FLAG fusion constructs, as well as OsNM3-FLAG as a nucleocytoplasmic-localized protein control (Shi et al., 2014), into N. benthamiana leaves. We performed subcellular fractionation analyses, followed by immunoblotting using FLAG antibody, accompanied by histone H3 antibody and phosphoenolpyruvate carboxylase (PEPC) antibody, which have been used as nuclear and cytosolic markers, respectively. The OsImp1a-FLAG and OsImp1b-FLAG proteins were detected in total protein extracts, the nucleus-enriched fraction and the nucleus-depleted fraction, in accordance with the nucleus and cytoplasm shuttle protein OsNM3 (Fig. 3A). Taken together, these data indicate that OsImp1a and OsImp1b are located in the nucleus and cytoplasm, suggesting their roles as cytoplasm/nuclear shuttle proteins to transfer proteins between the cytoplasm and nucleus.

Localization of OsImp1a and OsImp1b

To determine the expression patterns of OsImp1a and OsImp1b, we sampled the five tissues (root, stem, sheath, leaf and panicle) of IR24 at the booting stage, and assessed the transcript levels of the two genes in these tissues. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses showed that OsImp1a and OsImp1b were constitutively expressed in all of these tissues, with OsImp1a and OsImp1b having relatively higher transcription levels in the panicle compared with other tissues. OsImp1a showed slightly higher expression than OsImp1b in the different tissues (Fig. 3B).
We then tested the transcript accumulation of OsImpΔ1a and OsImpΔ1b in response to bacterial pathogen infection. OsImpΔ1a and OsImpΔ1b accumulation was rapidly and markedly induced after Xoo infection in the leaf tissue of IR24, which is susceptible to Xoo strain PXO99 (Fig. 3C). In addition, OsImpΔ1a and OsImpΔ1b transcription was significantly activated after Xoc infection (Fig. S4, see Supporting Information), suggesting that OsImpΔ1a and OsImpΔ1b transcription occurs in response to bacterial pathogen infection.

**OsImpΔ1a and OsImpΔ1b influence resistance to bacterial pathogens**

To determine whether OsImpΔ1a and OsImpΔ1b play a role in rice–bacterial pathogen interaction, OsImpΔ1a- and OsImpΔ1b-suppressing plants were generated using an RNA interference (RNAi) strategy. Because OsImpΔ1a and OsImpΔ1b showed similar expression patterns in diverse tissues and after bacterial pathogen infection, and had high nucleotide identity at 77% at the mRNA level (Fig. S5, see Supporting Information), it was difficult to choose a gene-specific fragment for the RNAi construct to target individual genes; therefore, we chose the common region of OsImpΔ1a and OsImpΔ1b to generate the RNAi construct to simultaneously suppress these two homologous genes. After the OsImpΔ1a/1b-RNAi construct had been transformed into IR24, which is susceptible to a large number of Xoo and Xoc strains, 11 independent transgenic plants were obtained. The expression of OsImpΔ1a and OsImpΔ1b was significantly reduced in 10 of the 11 plants. All of the transgenic plants were inoculated with Xoo strain PXO99 at the booting stage. The transgenic plants with lower OsImpΔ1a and OsImpΔ1b expression levels showed remarkably enhanced resistance to PXO99 with lesion lengths of $4.5 \pm 2.5$ cm to $8.8 \pm 2.7$ cm, compared with the wild-type lesion length of $21.8 \pm 1.1$ cm (Fig. 4). Increased resistance to Xoo was associated with reduced OsImpΔ1a and OsImpΔ1b expression, which was further confirmed in two T$_1$ families. T$_1$ families from two independent OsImpΔ1a/1b-RNAi T$_0$ plants with reduced expression of OsImpΔ1a and OsImpΔ1b were inoculated with Xoo at the booting stage. Some of the transgenic plants showed significantly enhanced resistance compared with the wild-type, and this resistance was associated with suppressed expression of OsImpΔ1a and OsImpΔ1b (Fig. S6, see Supporting Information). The reduction in Xoo-related disease symptoms in OsImpΔ1a/1b-RNAi plants was significantly correlated with the reduced expression of OsImpΔ1a and OsImpΔ1b in the OsImpΔ1a/1b-RNAi T$_1$ family ($r = 0.989$ for OsImpΔ1a, $r = 0.992$ for OsImpΔ1b, $n = 8$, $\alpha < 0.01$) and the OsImpΔ1a/1b-RNAi T$_1$ family ($r = 0.981$ for OsImpΔ1a, $r = 0.968$ for OsImpΔ1b, $n = 10$, $\alpha < 0.01$). Moreover, the Xoo growth rates in the leaves of OsImpΔ1a/1b-RNAi plants were observably lower than those in the wild-type (Fig. 5A). Furthermore, to assess whether OsImpΔ1a/1b-RNAi plants enhance resistance to other Xoo strains, we inoculated OsImpΔ1a/1b-RNAi7 and OsImpΔ1a/1b-RNAi8 T$_2$ families with 13 Xoo strains containing seven Philippine strains, four Chinese strains, one Japanese strain and one Korean strain, which are commonly used to test for broad-spectrum resistance to bacterial leaf blight. We found that OsImpΔ1a/1b-RNAi plants showed broad-spectrum resistance to the different Xoo strains (Fig. 5B).

In addition, we examined the resistance of OsImpΔ1a/1b-RNAi plants to the bacterial leaf streak pathogen Xoc. The RNAi lines and wild-type were inoculated with different Xoc strains at the tillering stage. The Xoc population was significantly lower in OsImpΔ1a/1b-RNAi plants than in the wild-type (Fig. 5C). The OsImpΔ1a/1b-RNAi plants exhibited clearly shorter lesion length than the wild-type after inoculation with different Xoc strains.
TALEs capture nuclear import receptors

(Fig. 5D). Taken together, these results suggest that OsImpα1a and OsImpα1b negatively regulate rice resistance to the bacterial pathogens Xoo and Xoc.

Activation of TALE-targeted susceptibility genes is inhibited in OsImpα1a/1b-RNAi plants

The bacterial pathogens Xoo and Xoc cause disease mainly through the targeting and activation of host rice susceptibility genes by their virulence TALEs. To monitor the expression pattern of rice TALE-targeted susceptibility genes on Xoo and Xoc infection in plants, we inoculated OsImpα1a/1b-RNAi plants with Xoo strain PX099 at the booting stage and Xoc strain RS105 at the tillering stage. qRT-PCR assays showed that the induced expression of the known susceptibility genes Xa13, OsTFIAY1 and OsTFX1, each of which is targeted by a different TALE of Xoo PX099, was significantly more suppressed in OsImpα1a/1b-RNAi plants than in wild-type plants (Fig. 6). Similarly, suppression of OsImpα1a and OsImpα1b in rice increased resistance to Xoc, which was associated with significantly hindered Xoc-activated expression of the rice susceptibility gene OsSULTR3;6, which is targeted by a TALE Tal2g of Xoc RS105, compared with wild-type plants (Fig. 6). These results demonstrate that the induction of TALE-targeted susceptibility genes is largely inhibited in OsImpα1a- and OsImpα1b-suppressing plants after bacterial pathogen infection.

Virulence of TALE-free and T3SS-free strains on OsImpα1a/1b-RNAi plants

To assess whether the ability of OsImpα1a and OsImpα1b to confer resistance to Xoo and Xoc is exclusively associated with the virulence factors of bacterial pathogens, TALEs, but not non-NALEs, we inoculated OsImpα1a/1b-RNAi plants at the booting stage with Xoo strain PX099 (TALE−), also named PH (Ji et al., 2016). The OsImpα1a/1b-RNAi plants showed similar lesion lengths to the wild-type (Fig. 7A), and the Xoo population was indistinguishable between leaves of OsImpα1a/1b-RNAi plants and the wild-type at 4 and 8 days after infection (Figs 7B, S7A, see Supporting Information). In addition, we inoculated
OsImpα1a/1b-RNAi plants and the wild-type at the booting stage with Xoo strain PXO99ΔhrcU, which is a T3SS-free strain lacking a functional T3SS and is unable to deliver TALEs and non-TALEs into plant cells (Guo et al., 2012). We found that there were similar Xoo populations in the leaves of OsImpα1a/1b-RNAi plants and the wild-type on the different days assessed after infection (Figs 7C, S7B). Furthermore, we simultaneously inoculated OsImpα1a/1b-RNAi plants and the wild-type at the tillering stage with Xoc strain RS105ΔhrcV, which is a T3SS-free strain lacking a functional T3SS (Guo et al., 2012). Similar Xoc populations were observed in the leaves of OsImpα1a/1b-RNAi plants and the wild-type on the different days evaluated after infection (Figs 7D, S7C). In brief, these data suggest that the broad-spectrum resistance of OsImpα1a/1b-RNAi plants to Xoo and Xoc relies on the existence of TALEs in bacterial pathogens.

**Mutational analysis of OsImpα1a/1b NLS binding sites**

A previous study has validated that OsImpα1a has two separate NLS binding sites, the major site and the minor site, both of which recognize positively charged amino acid clusters in NLSs (Chang et al., 2012). The residues D188 and E388 of OsImpα1a are essential for plant-specific NLS binding to the major site and the minor site, respectively (Chang et al., 2012). To investigate whether these two core residues of OsImpα1a are responsible for bacterial pathogen-derived NLS binding, we produced OsImpα1a derivatives with the key residues D188 and E388 substituted by positively or negatively charged amino acid residues. These
derivatives of OsImpα1 were co-transformed into yeast with Xoo-specific NLS to assess binding activity. Yeast two-hybrid assays showed that mutation in the major site D188 with positively charged amino acid residues (D188E) or negatively charged amino acid residues (D188H, D188K, D188R), and mutation in the minor site E388 with positively charged amino acid residues (E388D) or negatively charged amino acid residues (E388H, E388K, E388R), did not attenuate the interaction between OsImpα1a and NLS2 of pthXo1 (Fig. S8A, see Supporting Information). In addition, the residues D194 and E394 of OsImpα1b, the key amino acids for NLS binding to the major site and the minor site, respectively, were assessed for interaction with Xoo-derived NLS. Similarly, mutation in the major site D194 and in the minor site E394 did not influence OsImpα1b binding to Xoo-specific NLS (Fig. S8B). In conclusion, the mutations in the major site and minor site of rice OsImpα1a and OsImpα1b do not have a noticeable impact on the binding affinity for Xoo- or Xoc-derived NLS.

**DISCUSSION**

Previously, we have revealed that TALE-carrying *Xoo* and *Xoc* hijack the host basal transcription factor IIA γ subunit (TFIIAγ) in the plant nucleus to cause disease in rice (Yuan et al., 2016). Here, we further demonstrate that *Xoo* and *Xoc* capture host plant cytoplasm/nuclear shuttle proteins OsImpα1a and OsImpα1b for successful transfer of their virulence TALEs from the plant cytoplasm into the plant nucleus (Fig. 8). TALEs then interact with TFIIAγ, and bind and activate target susceptibility genes to cause disease in rice. This inference is supported by the following evidence. First, *Xoo* and *Xoc* employ their NLSs, specifically conserved NLS2, to interact with the host cytoplasm/nuclear shuttle proteins OsImpα1a and OsImpα1b. Second, transcriptional suppression of OsImpα1a and OsImpα1b enhances the resistance to diverse *Xoo* and *Xoc* strains, which is associated with the attenuated induction of susceptibility genes. Third, the efficient transportation of *Xoo* and *Xoc* TALEs into the plant nucleus is vital for bacterial pathogen invasion of rice.

The TALEs of isolated *Xoo* and *Xoc* strains with different geographical distributions all exclusively contain highly conserved NLS2, which is composed of five amino acid residues and is rich in arginine and lysine residues (RKRSR) (Booher et al., 2015; Cernadas et al., 2014). Although there are different amino acid residues of bacterial pathogen-derived NLS2 and plant nuclear proteins contained T-NLS and O2-NLS, which two have been proved selectively binding by both OsImpα1a and OsImpα1b (Jiang et al., 2001), TALEs of *Xoo* and *Xoc* mimic host nuclear proteins harbouring similar NLS for trapping by cytoplasm/nuclear shuttle proteins OsImpα1a and OsImpα1b, with the probably evidence that NLS2 of TALEs features with positively charged amino acids residues, lysine and arginine, which is accordance with T-NLS and O2-NLS. The exception to the transfer of plant-derived proteins from the cytoplasm into the nucleus, OsImpα1a, can directly bind to NLSs of *Agrobacterium tumefaciens* virulence protein VirD2, which forms a protein–nucleic acid supercomplex with T-DNA, facilitating T-DNA nuclear import (Chang et al., 2014). Moreover, the orthologues in pepper, CaImpα1 and CaImpα2, interact with TALE AvrBs3 of *Xcv* (Szurek et al., 2001). Thus, bacterial pathogens *Xoo* and *Xoc*, like *Xcv*, have evolved the simplest NLSs to mimic plant-derived nucleus-localized proteins for the facilitation of the transportation of virulence TALEs into the plant nucleus for the infection of host rice.

In rice, at least three nucleus importin α proteins have been characterized with nucleus trafficking capacity. We found that OsImpα1a and OsImpα1b, but not OsImpα2, interact with NLS2 of TALEs in *in vitro* and *in vivo* assays. The possible mechanism is that these three cytoplasm/nuclear shuttle proteins are responsible for the transportation of different nucleus-localized proteins by binding different types of NLS (Goldfarb et al., 2004). In order to identify whether there is functional redundancy of OsImpα1a and OsImpα1b for TALE transportation, we used the clustered regularly interspersed short palindromic repeats (CRISPR)/Cas system to separately knock out these two genes, with both homozygotes of the *osImpα1a* and *osImpα1b* mutants being developmentally lethal. An RNAi strategy was used to suppress the transcript of these two genes, and *OsImpα1a*/*1b*-RNAi plants showed slightly shorter flag leaf and panicle, fewer grains per panicle, decreased 1000-grain weight and significantly less seed setting than wild-type plants (Table S1, see Supporting Information). These data indicate that these two cytoplasm/nuclear shuttle proteins, OsImpα1a
and OsImpα1b, probably play comparable and pivotal roles in the transportation of NLS-containing proteins, which are essential for plant growth and development. Whether these two proteins play greater roles than their homologue OsImpα2 should be investigated further.

Rice importins α1a and α1b have been validated as components of the NLS receptor in plant cells, and transfer distinct groups of nuclear proteins (Jiang et al., 1998, 2001). Here, we found that these two importin proteins also carry bacterial pathogen-derived nuclear proteins into the plant nucleus. Our in vitro and in vivo assays showed that OsImpα1a and OsImpα1b associated with TALEs are dependent on the presence of NLS2, but not NLS1 or NLS3, which is in accordance with the analysis of TALE AvrBs3 of Xoo, where deletion of NLS1 or NLS3 of AvrBs3 is not sufficient to abolish the induction of the AvrBs3-activated hypersensitive response (HR) on B5-containing resistant pepper plants (Szurek et al., 2001). NLS1, NLS2 and NLS3 of Xanthomonas are rich in positively charged amino acid residues. However, Xanthomonas species selectively use their NLS2, not NLS1 or NLS3, of TALEs to trap host plant cytoplasm/nuclear shuttle proteins. The functions of NLS1 and NLS3 during the process of Xanthomonas TALE-triggered susceptibility in plants requires further study.

Both OsImpα1a and OsImpα1b were induced in rice leaves in response to infection with the bacterial pathogens Xoo or Xoc, whereas their homologous genes in pepper, Caimpα1 and Caimpα2, were constitutively expressed independent of infection with Xcv (Szurek et al., 2001). This diversity could be caused by differences between the respective host plants, rice and pepper. Plants with suppressed OsImpα1a and OsImpα1b transcription showed broad-spectrum disease resistance to Xoo and Xoc, accompanied by attenuated induction of susceptibility genes. The reason that TALEs targeting susceptibility genes could not be immediately up-regulated after Xoo and Xoc infection is a result of the inefficient transportation of TALEs into the plant nucleus. However, OsImpα1a/1b-RNAi plants showed a similar lesion length and bacterial population to the wild-type after inoculation with TALE-free and T3SS-free bacterial strains. These results further indicate that suppression of OsImpα1a or OsImpα1b-mediated resistance to Xoo or Xoc is solely associated with TALEs, the major virulence factors of bacterial pathogens.

In conclusion, the present results and a previous report (Szurek et al., 2001) suggest that the TALE-carrying bacterial pathogens, Xoo, Xoc and Xcv, use the same mechanism, i.e. the trapping of host plant nuclear import receptor proteins, to transfer their virulence TALEs from the plant cytoplasm into the nucleus to activate susceptibility genes to cause disease in rice and pepper. The TALE-carrying genus Xanthomonas infects a wide range of plants (Jacques et al., 2016), and the virulence TALEs contain an identical NLS2. The plants simultaneously carry nuclear import receptors with high amino acid sequence similarity (Wiermer et al., 2007). Thus, moderate suppression of expression of plant nuclear import receptor proteins may provide an applicable strategy to improve disease resistance to TALE-carrying bacterial pathogens in other plants.

**EXPERIMENTAL PROCEDURES**

**Plant and bacterial materials**

Rice (Oryza sativa ssp. Xian) IR24 is susceptible to Xoo and Xoc, and was used in this study. Plants were grown during a normal rice-growing season under natural field conditions.

The reference Xoo strain PXO99A and its mutation PH [PXO99A (TALE–)], with deletion of all 19 TALE genes, and Xoc strain RS105 have commonly been used in studies of rice resistance to bacterial leaf blight disease and bacterial leaf streak disease, respectively (Ji et al., 2016), and were used in this study. Xoo strain PXO99 was used for pathogen inoculation. PXO99A, a 5-azacytidine-resistant mutant of PXO99, was used for genetic manipulation and pathogen inoculation. All the Xanthomonas strains were grown at 28 °C on nutrient agar medium. When genetic manipulation of bacteria was undertaken, antibiotics were used at the following final concentrations as required: ampicillin, 100 μg/mL; rifampicin, 75 μg/mL; kanamycin, 25 μg/mL.

**Site-directed mutation**

Site mutation of plant OsImpα1a and OsImpα1b genes and Xoo pthXo1 gene was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen Life Technologies, Carlsbad, CA, USA), as described previously (Yuan et al., 2009). The site-mutated genes were confirmed by Sanger sequencing.

**Vector construction and plant transformation**

The TALE pthXo1 and its variations were cloned into the pHM1 vector to produce pHM1-pthXo1, and then transferred into Xoo strain PH following a published method (Ji et al., 2016). The NLS2 region of pthXo1 was replaced with its site-directed mutations by Gibson assembly (Gibson et al., 2009), following confirmation by Sanger sequencing.

The full-length cDNAs of OsImpα1a, OsImpα1b and OsNMD3 were ligated into the pU1301-3FLAG vector. The recombinant vectors were introduced into A. tumefaciens strain GV3101. Agrobacterium-mediated transformation was performed by infiltration into N. benthamiana leaves using a needleless syringe (Yuan et al., 2016).

To construct the RNAi vector, the gene-specific and high-similarity fragment of OsImpα1a and OsImpα1b genes was amplified and inserted into the pDS1301 vector (Yuan et al., 2010). The recombinant vector was introduced into A. tumefaciens strain EHA105. Agrobacterium-mediated transformation was performed using calli derived from mature embryos of rice variety IR24, according to a published protocol (Ge et al., 2006).
Protein–protein interaction

To study the interaction between Xanthomonas TALEs and rice OsImpα1a, OsImpα1b and OsImpα2 in yeast two-hybrid assays, the different domains of TALE pthXo1 and its variations were amplified using gene-specific primers (Yuan et al., 2016); the amplified DNA segments were then ligated into the pGBK7 vector. Rice OsImpα1a, OsImpα1b, OsImpα2 and their variations were amplified using gene-specific primers (Table S2, see Supporting Information), and the amplified DNA segments were ligated into the pGADT7-Rec vector. The recombinant pGBK7 and pGADT7 plasmids were then co-transformed into yeast strain AH109 for interaction analyses (Yuan et al., 2010). The yeast clones were restreak on synthetic defined premixed (SD) medium lacking leucine (L) and tryptophan (W) (−LW) and selective SD medium lacking L, W, histidine (H) and adenine (A) (−LWHA).

To study the interaction in planta, Co-IP assays were performed (Yuan et al., 2016). The DNA segments of pthXo1 and its variations were ligated into the pU1301-9myc vector; the DNA segments of OsImpα1a, OsImpα1b and OsImpα2 were ligated into the pU1301-9myc vector (Yuan et al., 2010). The recombinant constructs were introduced into A. tumefaciens strain GV3101 by electroporation. Agrobacterium-mediated transformation was performed by infiltration into N. benthamiana leaves using a needleless syringe. Co-IP assays were carried out using anti-FLAG antibody (F7425, Sigma, Sigma-Aldrich, St. Louis, Missouri, USA) and anti-myc antibody (AB103, Tiangen, Beijing, China), as described previously (Yuan et al., 2016). Each Co-IP assay was repeated at least twice.

Split-luciferase complementation assay

To construct split-luciferase complementation assay vector, the open reading frame of pthXo1 was inserted into the vector pCAMBIA-35S-nLUC to generate construct pthXo1-nLUC; the open reading frames of OsImpα1a, OsImpα1b and OsImpα2 were inserted into the vector pCAMBIA-35S-cLUC to generate constructs OsImpα1a-cLUC, OsImpα1b-cLUC and OsImpα2-cLUC, respectively. The recombinant constructs were introduced into A. tumefaciens strain GV3101. Equal amounts of Agrobacterium cultures for nLUC and cLUC constructs were infiltrated into intercellular spaces of fully expanded rice leaves with needleless syringes at three different locations per leaf. The bacterial growth rate in rice leaves was determined by counting the number of colony-forming units (Yuan et al., 2016).

For the measurement of the growth in planta of Xoo strains PXO99AΔhrcV, these pathogens were infiltrated into the intercellular spaces of fully expanded rice leaves with needleless syringes at three different locations per leaf. The bacterial growth rate in rice leaves was determined by counting the number of colony-forming units (Yuan et al., 2016).

Gene expression analysis

For gene expression analysis, real-time qRT-PCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, California, USA). In brief, 2-cm rice leaf fragments near the bacterial infection sites were collected for RNA isolation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA). An aliquot (5 μg) of total RNA was treated with RNase-free DNase I (Invitrogen) to remove potentially contaminating DNA, and first-strand cDNA was reverse transcribed from total RNA with oligo(dT)18 primer using M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. qRT-PCR was conducted using gene-specific primers (Table S3, see Supporting Information). The expression level of the rice actin gene was used to standardize the RNA sample as an internal control. The expression level relative to that of controls was assessed. Each qRT-PCR assay was repeated at least twice with a similar result, with each repetition having three replicates.

Western blotting

The nuclear proteins were extracted as described previously (Moes et al., 2013). The protein samples were separated on a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto a nitrocellulose membrane and then analysed by blotting with different antibodies. The antibodies used for immunoblotting analyses included anti-FLAG.
(F7425, Sigma), anti-Histone H3 (06-755, Millipore, Burlington, Massachusetts) and anti-PEPC (AS09458, Agrisera, Vannas, Sweden).

Sequence analysis

Multiple sequence alignments of amino acid sequences were generated using CLustalW in MEGA X with the default parameters. The sequence alignments obtained were used as input for the neighbour-joining analysis in MEGA X to construct the phylogenetic tree. For phylogenetic tree construction, a bootstrap method with 1000 replications was used for test of phylogeny, with Poisson model and pairwise deletion during gaps/missing data treatment.

Statistical analysis

Differences between samples were analysed for statistical significance using SPSS software and Student’s t-test (two-tailed). The correlation analysis between disease symptom and gene expression level was analysed using the Pearson correlation coefficient analysis in SPSS (IBM SPSS Statistics, Version 19.0, IBM Corp., released 2010, Armonk, New York).

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### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1** Phylogenetic tree of plant importin α proteins. Sequences were analysed by the neighbour-joining method with genetic distance calculated by MEGA X. *OsImp2α* (XP_015621115), *OsImp1b* (XP_015639761) and *OsImp2* (XP_015619230) from *Oryza sativa*; *Calmp2α* (AAK38726) and *Calmp2α* (AAK38727) from *Capsicum annum*; LeKAP1 (AAC23722) from *Solanum lycopersicum*; AtImp1α (NP_187328), AtImp2α (NP_001154239), AtImp3α (NP_192124) and AtImp4α (NP_172398) from *Arabidopsis thaliana*; *CsImp1α* (XP_006480039), *CsImp1b* (XP_006472169) and *CsImp2α* (XP_006488879) from *Citrus sinensis*; *GhImp1α* (XP_016667887) and *GhImp2α* (XP_016695962) from *Gossypium hirsutum*; *PvImpα* (XP_007147298) from *Phaseolus vulgaris*.

**Fig. S2** NLS2 of *pthXo1* interacts with rice *OsImp1a* and *OsImp1b* in yeast cells by yeast two-hybrid assay. The interactions were assessed by the growth of yeast cells on synthetic defined premixed (SD) medium lacking (−) leucine (L), tryptophan (W), histidine (H) and adenine (A). Vector, empty vector as control; RR, repeat region; TFB, transcription factor binding region; NLS, nuclear localization signal. (A) NLS2 of *pthXo1* interacts with *OsImp1a*. (B) NLS2 of *pthXo1* interacts with *OsImp1b*. (C) NLS2 of *pthXo1* does not interact with *OsImp2α*.

**Fig. S3** Interaction between point-mutated NLS2 of *pthXo1* and *OsImp1b* analysed by yeast two-hybrid assay. The interactions were assessed by the growth of yeast cells on synthetic defined premixed (SD) medium lacking (−) leucine (L), tryptophan (W), histidine (H) and adenine (A). Vector, empty vector as control; RR, repeat region; TFB, transcription factor binding region; NLS, nuclear localization signal. (A) *NLS2 of pthXo1 interact with OsImp1a*. (B) *NLS2 of pthXo1 interact with OsImp1b*. (C) *NLS2 of pthXo1* does not interact with *OsImp2α*.

**Fig. S4** Expression of *OsImp1a* and *OsImp1b* after infection with *Xanthomonas oryzae pv. oryzicola* (*Xoc*) strain RH3 at the tillering stage.

**Fig. S5** Nucleotide sequence alignment of *OsImp1a* and *OsImp1b* by MUSCLE.
(T3SS-free strain) at the booting stage, or *Xanthomonas oryzae pv. oryzicola* (Xoc) strain RS105ΔhrcV (T3SS-free strain) at the tillering stage. Data represent the mean (nine leaves from three plants) ± standard deviation (SD). (A) Growth of *Xoo* strain PXO99Δ (TALE) in leaves of OsImpα1a/1b-RNAi8 plants. (B) Growth of *Xoo* strain PXO99ΔhrcU in leaves of OsImpα1a/1b-RNAi8 plants. (C) Growth of *Xoc* strain RS105ΔhrcV in leaves of OsImpα1a/1b-RNAi8 plants.

**Fig. S8** Interaction between NLS2 of pthXo1 and point-mutated OsImpα1a (A) or OsImpα1b (B) analysed by yeast two-hybrid assay. The interactions were assessed by the growth of yeast cells on synthetic defined premixed (SD) medium lacking (–) leucine (L), tryptophan (W), histidine (H) and adenine (A).

**Table S1** Measurements of agronomic traits of OsImpα1a/1b-RNAi plants under natural field conditions

**Table S2** Polymerase chain reaction (PCR) primers used for the construction of vectors for transformation and protein–protein interactions.

**Table S3** Polymerase chain reaction (PCR) primers used for quantitative reverse transcription (RT)-PCR assays.