Molecules and Cells

Pi5 and Pii Paired NLRs Are Functionally Exchangeable and Confer Similar Disease Resistance Specificity

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Effector-triggered immunity (ETI) is an effective layer of plant defense initiated upon recognition of avirulence (Avr) effectors from pathogens by cognate plant disease resistance (R) proteins. In rice, a large number of *R* genes have been characterized from various cultivars and have greatly contributed to breeding programs to improve resistance against the rice blast pathogen *Magnaporthe oryzae*. The extreme diversity of *R* gene repertoires is thought to be a result of co-evolutionary history between rice and its pathogens including *M. oryzae*. Here we show that *Pii* is an allele of *Pi5* by DNA sequence characterization and complementation analysis. *Pii-1* and *Pii-2* cDNAs were cloned by reverse transcription polymerase chain reaction from the *Pii*-carrying cultivar *Fujisaka5*. The complementation test in susceptible rice cultivar *Dongjin* demonstrated that the rice blast resistance mediated by *Pii*, similar to *Pi5*, requires the presence of two nucleotide-binding leucine-rich repeat genes, *Pii-1* and *Pii-2*. Consistent with our hypothesis that *Pi5* and *Pii* are functionally indistinguishable, the replacement of *Pii-1* by *Pi5-1* and *Pii-2* by *Pi5-2*, respectively, does not change the level of disease resistance to *M. oryzae* carrying AVR-Pii. Surprisingly, Exo70F3, required for *Pii*-mediated resistance, is dispensable for *Pi5*-mediated resistance. Based on our results, despite similarities observed between Pi5 and Pii, we hypothesize that Pi5 and Pii pairs require partially distinct mechanisms to function.

Keywords: allelism, *Magnaporthe oryzae*, *Pi5*, *Pii,* resistance, rice

INTRODUCTION

Pathogen infection causes tragic damage to host plants and serious reduction of crop yield. Resulting from their co-evolution, pathogens and plants have developed efficient mechanisms to evade detection and activate disease resistance, respectively (Huang et al., 2014). Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) have emerged as two key immunity layers in plants. Whereas PTI is associated with the perception of PAMPs by pattern recognition receptors (PRRs),

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ETI is the second tier of immunity where the plant recognizes secreted effector proteins which, in the absence of resistance proteins, might dampen PTI and benefit the pathogen (Jones and Dangl, 2006). ETI is often associated with rapid development of programmed cell death at the site of infection termed the hypersensitive response (HR) (Maekawa et al., 2011), a strong resistance mechanism that prevents microbial proliferation. The specific recognition of pathogen-derived avirulence (Avr) effectors by corresponding disease resistance (R) proteins activates ETI in plants. R proteins typically carry nucleotide-binding (NB) and leucine-rich repeat (LRR) (NLR) domains with varying N-terminal toll/interleukin 1 or coiled-coil domains. Recently, it was discovered that some NLRs carry effector-sensing decoy domains at their C-terminal regions (Cui et al., 2015).

In order to understand the mechanism of disease resistance to the rice blast fungus *Magnaporthe oryzae*, many PRRs, NLRs and resistance-associated quantitative trait loci were identified and functionally investigated using monogenic rice lines (Inukai et al., 1996; Jia and Liu, 2011; Mackill, 1992; Tsunematsu et al., 2000). In particular, most of the cloned genes encode NLR proteins (Singh et al., 2015; Wang et al., 2014). In addition, various mechanisms by which rice NLRs recognize corresponding Avr effectors have been proposed (Wu et al., 2014; Zhang et al., 2013). These include Pi-ta-dependent direct recognition of the *M. oryzae* Avr effector, AVR-Pita (Jia et al., 2000) and indirect interaction of Piz-t and AVR-Piz-t (Park et al., 2016). More recently, the paired NLRs, RGA4 and RGA5, were shown to function together in recognition of the *M. oryzae* Avr effectors AVR1-CO39 and AVR-Pia (Césari et al., 2014). Although the mechanistic basis differs between each other, these recognition events activate downstream defense signaling and efficiently restrict pathogen proliferation in rice.

Despite the effective disease resistance against blast fungus conferred by NLRs in rice, the rapid onset of genetic variability and pathogenicity of blast fungus has led to the disarmament of resistance conferred by specific *R* genes. Hence, plants have evolved a repertoire of *R* genes to counter pathogen arsenals, as evidenced by sequence polymorphisms in various cultivars (Chisholm et al., 2006; Jacob et al., 2013; McDonald and Linde, 2002; Schulze-Lefert and Panstruga, 2011). In several cases, allelic sequence diversity of NLR genes have caused functional variation, especially in regards to Avr recognition. For instance, the *Pi2/Pi9* locus contains six NLR-coding genes, of which three cloned genes, *Pi2*, *Pi9*, and *Piz-t*, confer different resistance specificities. Further study indicated that the difference is the result of a few amino acid changes (Wu et al., 2012). Among 20 sequence polymorphisms detected in 24 *Pita* natural variants, only the 918th amino acid is critical for AVR-Pita recognition (Bryan et al., 2000; Wang et al., 2016).

Pi5 and *Pii* genes were identified in a number of rice cultivars. The similarity of resistance specificity conferred by *Pii* and *Pi5* was reported previously (Ebron et al., 2004; Selisana et al., 2017). Consistently, in polymerase chain reaction (PCR) analysis of 24 different monogenic lines, we found that *Pi5*-specific markers are present only in *Pi5*-, *Pi3*-, and *Pii*-carrying lines (Yi et al., 2004). In a separate study, *Pi3* was shown to be identical to *Pi5* based on DNA gel-blot and genomic sequence analyses (Jeon et al., 2003). The *Pi5* gene was identified and cloned from *RIL260,* a known rice cultivar resistant to *M. oryzae* isolates collected from Korea and the Philippines, by a map-based cloning method (Lee et al., 2009). Interestingly, it was shown that *Pi5*-mediated resistance requires two adjacently located NLR genes, *Pi5-1* and *Pi5-2,* which was demonstrated by coexpressing their genomic DNA fragments in the susceptible cultivar *Dongjin*. Therefore, we hypothesized that *Pii*-mediated resistance requires a pair of NLR genes that are allelic to those of *Pi5*. Here in this work, we cloned the *Pii* resistance genes and analyzed the allelism relationship by complementation test.

MATERIALS AND METHODS

Genomic DNA PCR and reverse transcription PCR (RT-PCR) analysis

Twelve PCR primer sets (Supplementary Table S1) were designed across the *Pi5-1* and *Pi5-2* genomic DNA sequences to amplify the counterparts in *Pii*-carrying cultivar *Fujisaka5*. PCR amplification was performed in a final volume of 40 μl. The Taq DNA polymerase (BIOFACT, Korea) was used according to the manufacturer's instructions. The PCR product purified was sequenced. The whole sequence of *Pii* was assembled from the fragments sequenced separately and aligned to *Pi5* sequences using the ClustalW2 program.

To analyze gene expression, total RNA from leaf blades of four-week-old transgenic plants was extracted using RNAiso Plus according to the manufacturer's protocol (Takara Bio, Japan). Reverse transcription was performed according to the manufacturer's protocol using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The cDNA obtained was used as template to measure expression by quantitative PCR (qPCR) with gene-specific primers (Supplementary Table S1). Three biological replications were carried out for each sample.

Vector construction and generation of transgenic rice plants

Full-length cDNAs of *Pi5-1* and *Pi5-2*, and *Pii-1* and *Pii-2* were isolated by RT-PCR from leaves of *RIL260* carrying *Pi5* and *Fujisaka5* carrying *Pii*, respectively. The cDNAs subcloned into pJET1.2 (Thermo Scientific, USA) were transferred to the plant overexpression vectors pPZP2Ha3 (Fuse et al., 2001), pGWB514 and pGWB518 (Nakagawa et al., 2007), and a modified pCAMBIA3301 under the control of CaMV35S promoter (35S). To construct the RNAi vectors, unique sequences of *Pi5-1* and *Pi5-2* were amplified by PCR with gene-specific primers (Supplementary Table S1) and inserted into the pANDA vector with the antisense sequence upstream of the sense one as described (Miki and Shimamoto, 2004). To create *OsExo70F3* mutants, a designed guide RNA (5′-TCCCATCACCCCGCACAGCC-3′) was cloned into an entry vector, pOs-sgRNA. The resulting sgRNA expression cassette was transferred into pH-Ubi-cas9-7 to construct the *Exo70F3* CRISPR/Cas9 vector (Miao et al., 2013).

To produce transgenic rice plants, the *Agrobacterium tumefaciens* LBA4404 strains harboring the constructed vectors

were grown for three days at 28°C, and transgenic calli were obtained via the *Agrobacterium*-mediated co-cultivation method as described previously (Jeon et al., 2000). *Pi5-1*, *Pi5-2* and *Pii-1* cDNA constructs were introduced to a susceptible cultivar *Dongjin* using hygromycin selection. *Pii-2* and *Pii-2*Δ cDNA constructs were introduced into *Pii-1* cDNA transgenic rice using phosphinothricin selection as a second selectable marker. The *Exo70F3* CRISPR/Cas9 construct was transformed to transgenic *Dongjin* plants coexpressing *Pi5-1* and *Pi5-2* genomic DNAs using phosphinothricin selection (Lee et al., 2009). Transgenic rice plants were selected on a medium containing 50 mg • L^{-1} hygromycin or 10 mg • L^{-1} phosphinothricin and 250 mg • L^{-1} cefotaxime.

To identify *OsExo70F3* mutants, PCR amplification with the primers (Supplementary Table S1) specific to the target PAM region was performed with genomic DNAs of primary transgenic plants. The PCR product purified was sequenced to determine mutations created by the CRISPR/Cas9 system (Miao et al., 2013). Full-length cDNAs of *OsExo70F3* were amplified from all *OsExo70F3* mutants by RT-PCR (Supplementary Table S1) and sequenced to confirm the mutations.

Pathogen inoculation

For blast fungus inoculation, *M. oryzae* isolate PO6-6 was cultured on V8 juice agar plates (80 ml V8 Juice [Campbell's Soup Company, USA] L^{-1}, 15 g agar L^{-1}, pH 6.8) for two weeks under fluorescent light. Subsequently, spores were collected and suspended in water to reach a concentration of 5×10^6 ml^{-1} for spot inoculation and 2×10^5 ml^{-1} for spray inoculation. Fully expanded healthy leaf regions, without any cell death lesions, of six-week-old and four-week-old plants, respectively, were inoculated with the spore suspensions prepared for spot inoculation and spray inoculation (Kanzaki et al., 2002). Leaf samples were collected and photographed to evaluate blast lesions when disease symptoms were well developed in susceptible controls. Three replications were carried out for each sample.

RESULTS

Molecular cloning of *Pii* cDNAs

In order to verify the pair of NLRs as orthologues of *Pi5-1* and *Pi5-2* genomic sequence, we used twelve sets of primers, designed based on *Pi5-1* and *Pi5-2* genome sequences (Fig. 1A and Supplementary Table S1), and amplified the respective PCR fragments from genomic DNA of a rice cultivar *Fujisaka5* known to show *Pii*-mediated resistance (Fig. 1B). All primer pairs did not amplify PCR products in the susceptible cultivar, *Dongjin*, except for the Pi5-1 F5/R5 primer set which could amplify a genomic sequence similar to a part of Pi5-1 C-terminal region from *Dongjin* (which is also conserved in the genome of susceptible cultivar *Nipponbare*). DNA sequence analysis of PCR products showed that the *Pi5* counterparts in *Fujisaka5* are highly similar to *Pi5* genes (Lee et al., 2009). Meanwhile, an independent study deployed the Mutmap-Gap method to characterize the *Pii* locus, suggesting the presence of a *Pi5* allele in cultivar *Hitomebore* (Takagi et al., 2013). The resistance gene *Pii* was isolated in the mutant lines that have lost *Pii* function. Nucleotide alignment analysis denoted the identity of the previously identified *Pii* gene (Takagi et al., 2013) with our *Pi5-1* counterpart in *Fujisaka5*, herein referred to as *Pii-1* (GenBank accession No. MH490982), whereas the other gene is referred to as *Pii-2* (GenBank accession No. MH490983) in our study. In order to verify cDNA sequences of *Pii-1* and *Pii-2* in *Fujisaka5*, *Pii* cDNAs were cloned by RT-PCR using cDNA prepared from the leaves infected with *M. oryzae* PO6-6, and subjected to sequence analysis. Six and 11 polymorphic amino acids were

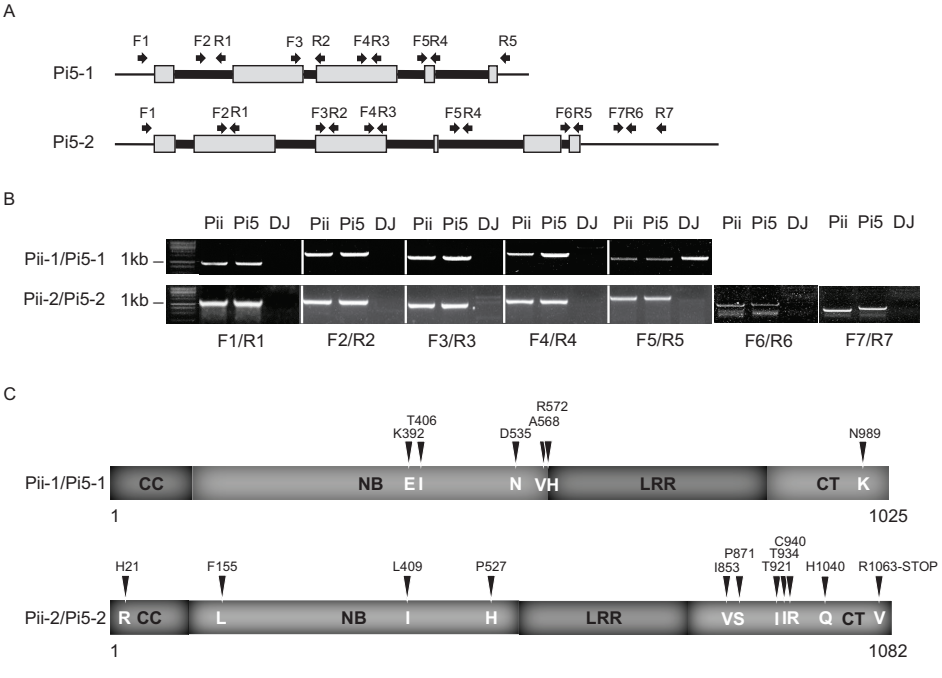

Fig. 1. Comparison of *Pii* and *Pi5* sequences. (A) Primers (black arrows) used for amplifying *Pii* in *Fujisaka5*. Grey box and black box indicate exons and introns, respectively. (B) PCR results from *Fujisaka5* genomic DNA using primers shown in (A). (C) Amino acid differences between Pii (*Fujisaka5*) and Pi5. Letter and numbers above downward triangles indicate the amino acids in Pi5 and position corresponding to the white letters in the Pii protein schematic. Each domain in each gene is presented as rectangles. DJ, *Dongjin* (susceptible cultivar); CC, coiled-coil; NB, nucleotide binding site; LRR, leucine-rich repeat; CT, C-terminal domain.

confirmed in *Pii-1* and *Pii-2* from cultivar *Fujisaka5* as compared to *Pi5-1* and *Pi5-2*, respectively (Fig. 1C). Additionally, *Pii-2* contains an extended C-terminus of 20 amino acids, in contrast to *Pi5-2*. DNA sequence analysis suggested that *Pii-2* and *Pi5-2* might have different splicing patterns due to significant variation in the last intron (Supplementary Fig. S1).

To test if Pii-1 and Pii-2 are required for AVR-Pii recognition in *Fujisaka5*, RNA interference (RNAi)-mediated knockdown of *Pii-1* or *Pii-2* transcripts were induced by introducing RNAi constructs of *Pi5-1* or *Pi5-2* into a *Pii* monogenic line (IRBLi-F5) that carries both *Pii-1* and *Pii-2* derived from a cross between *Fujisaka5* and susceptible background cultivar *Lijiangxintuanheigu* (Tsunematsu et al., 2000) (Fig. 2A). Expression of *Pii-1* or *Pii-2* was significantly decreased in *Pii-1/Pi5-1* or *Pii-2/Pi5-2* RNAi lines, respectively, as evaluated by quantitative RT-PCR in the selected transgenic lines (Fig. 2B). To test whether the reduction in *Pii-1* or *Pii-2* expression correlates with reduced disease resistance/AVR-Pii recognition, we challenged wild-type IRBLi-F5 and RNAi transgenic lines with *M. oryzae* PO6-6, which carries AVR-Pii and AVR-Pi5 (Kobayashi et al., 2007), using a spray inoculation method. Distinct lesions developed at seven days post-infection (dpi) in transgenic plants (Fig. 2C), suggesting that the resistance by *Pii* was abolished due to the reduced expression of *Pii-1* or *Pii-2*. This result indicates that both *Pii-1* and *Pii-2* are required for AVR-Pii-triggered resistance to *M. oryzae*.

Functional evaluation of *Pi5* and *Pii* cDNAs in transgenic rice

To further investigate whether the Pii-1/Pii-2 pair is required, we generated a series of binary constructs carrying *Pi5* or *Pii* variants whose expression is controlled by the 35S promoter (Fig. 3A). *Pi5* constructs were transformed separately to a susceptible rice cultivar *Dongjin,* in which the *Pi5* allelic sequence is absent (Figs. 3B and 3C). While transgenic lines expressing *Pi5-1* or *Pi5-2* cDNA displayed wild-type morphology, transgenic plants expressing both *Pi5-1* and *Pi5-2* cDNAs developed by crossing the transgenic lines carrying *Pi5-1* and *Pi5-2* displayed an autoimmune lesion mimic (LM) phenotype. The phenotype was characterized by cell death areas arising from the leaf tip and apparently seen in the third leaf at five weeks of age under paddy field conditions in the summer (Supplementary Fig. S2). To validate the cloned *Pi5-1* and *Pi5-2* cDNAs in disease resistance to blast fungus, the transgenic plants expressing *Pi5-1* and/or *Pi5-2* cDNAs were spot inoculated with *M. oryzae* PO6-6 (Figs. 3B and 3C). A susceptible control, *Dongjin*, as well as transgenic lines expressing *Pi5-1* or *Pi5-2* cDNA displayed long brown lesion at nine dpi, characteristic of a compatible interaction. Importantly, the transgenic lines carrying genomic sequences (Lee et al., 2009) or cDNAs of both *Pi5-1* and *Pi5-2* were resistant to *M. oryzae* PO6-6, demonstrating that the cloned cDNAs of *Pi5-1* and *Pi5-2* are functional.

In order to verify if the transgenic plants expressing *Pii-1* and/or *Pii-2* show similar disease resistance/susceptibility phenotypes to *Pi5*-expressing lines, susceptible *Dongjin* plants were first transformed with *Pii-1* cDNA. The possibility of two spliced forms of *Pii-2* due to the significant difference in the last intron of *Pii-2* and *Pi5-2* (Supplementary Fig. S1) led us to investigate the function of *Pii-2* cDNAs both with a longer C-terminus and without it (*Pii-2Δ*). The *Pii-2Δ* variant lacks the last exon and therefore has a C-terminal region identical to *Pi5-2* (Fig. 3A). *Pii-2* or *Pii-2Δ* cDNA was then introduced to *Pii-1* cDNA-expressing transgenic *Dongjin*. Each of the five transgenic plants with different expression of each transgene was evaluated for susceptibility (Supplementary Fig. S3). While transgenic lines expressing only *Pii-1* cDNA were susceptible, all lines expressing *Pii-2* or *Pii-2Δ* cDNA additionally

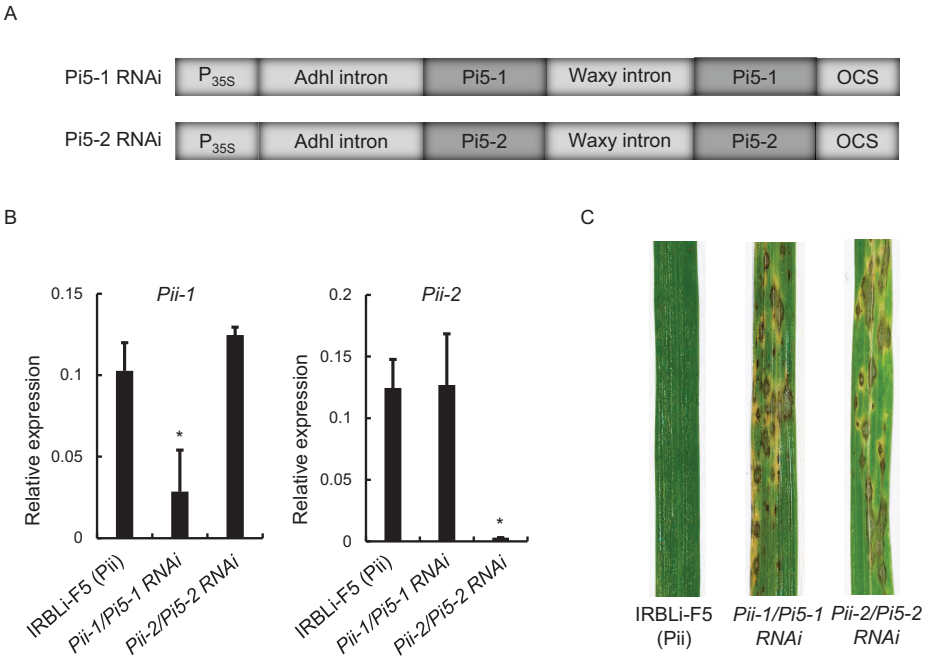

Fig. 2. Characterization of transgenic rice plants with reduced expression of *Pii-1* or *Pii-2*. (A) Illustration of *Pi5-1/Pii-1* and *Pi5-2/Pii-2 RNAi* constructs used for transformation to *Pii*-carrying line, *IRBLi-F5*. (B) Quantitative RT-PCR analysis of *Pii-1* or *Pii-2* in RNAi lines. The rice gene *Ubiquitin5* (*OsUbi5*; LOC_Os01g22490) was used to normalize gene expression. Asterisks represent statistical significance with Student's *t*-test, $P < 0.001$. (C) Disease symptoms on the mature leaves of three to four-week-old plants photographed at seven dpi with rice blast isolate, *M. oryzae* PO6-6 carrying AVR-Pii.

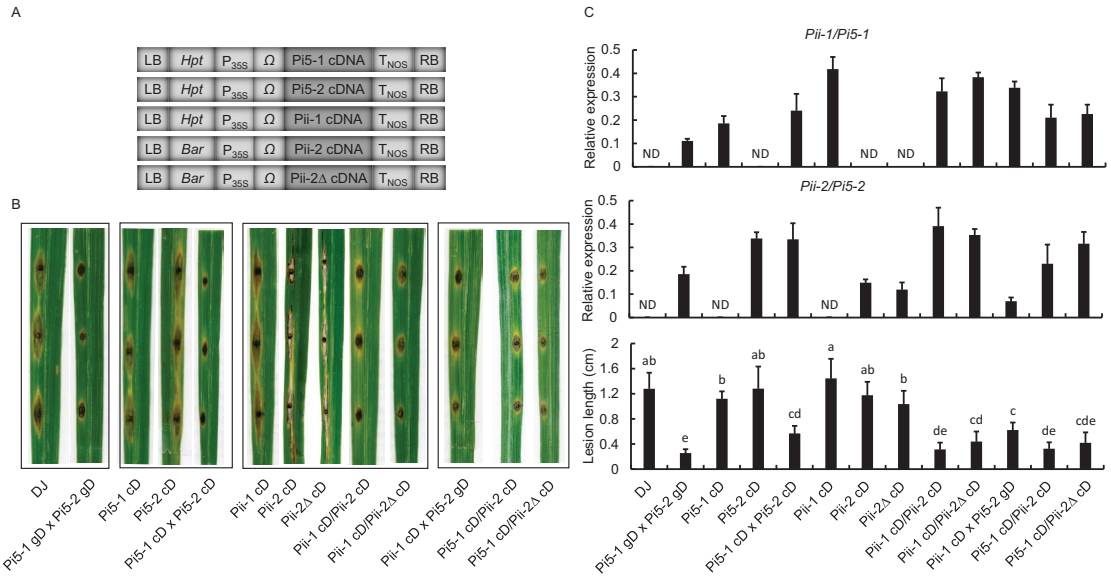

Fig. 3. Characterization of transgenic rice plants expressing *Pii* and *Pi5* genes. (A) Schematic representation of *Pi5* and *Pii* cDNA-overexpression binary constructs. (B) Representative leaves of the six-week-old transgenic plants carrying indicated transgene(s) at nine dpi with *M. oryzae* PO6-6. (C) Expression of *Pii-1/Pi5-1* and *Pii-2/Pi5-2* evaluated by quantitative RT-PCR analysis (upper panels) and quantitative data of disease lesion lengths (lower panel). The rice gene *Ubiquitin5* (*OsUbi5*; LOC_Os01g22490) was used to normalize gene expression. ND, not detected; DJ, *Dongjin* (susceptible cultivar); cD, cDNA; gD, genomic DNA. Data analyzed by ANOVA (α = 0.05), and Tukey's honestly significant difference (HSD) test was used to conduct mean separation. Different letters indicate significant difference.

were resistant to *M. oryzae* PO6-6 (Figs. 3B and 3C). In addition, the transgenic plants expressing *Pii-2* or *Pii-2Δ* without *Pii-1* were susceptible (Figs. 3B and 3C). This result showed that *Pii*-mediated resistance is similar to *Pi5*, consistent with our hypothesis that *Pii* and *Pi5* display similar recognition specificity. Interestingly, we also observed the LM phenotype in some transgenic plants expressing *Pii-2* or *Pii-2Δ* cDNA in the presence of *Pii-1* cDNA at the late stage of development (Supplementary Fig. S3), which is consistent with the observation from *Pi5* transgenic rice plants. To confirm that this resistance is *Pii*- and *Pi5*-mediated, we challenged all the transgenic plants with a compatible *M. oryzae* KI215 isolate lacking AVR-Pii and AVR-Pi5 (Lee et al., 2009). As expected, the lesions developed at nine dpi in all the transgenic plants were comparable to the levels of the susceptible cultivar *Dongjin* (Fig. 4).

Genetic swapping between *Pii* and *Pi5* paired NLR counterparts

The high similarity of the *Pii*- and *Pi5*-paired *R* genes further prompted us to test the combination of *Pii* and *Pi5* genes. Therefore, we generated transgenic lines expressing *Pii-1* and *Pi5-2* by crossing a *Pii-1* cDNA overexpressing line to the line expressing *Pi5-2* genomic DNA. The resulting plants thus expressed both *Pii-1* and *Pi5-2* (Fig. 3C). In addition, we similarly generated transgenic plants expressing *Pii-2* or *Pii-2Δ* in the presence of *Pi5-1* cDNA by introducing *Pii-2* or *Pii-2Δ* cDNAs to *Pi5-1* cDNA expressing line. Surprisingly, the crossed plants showed clear incompatible interaction with *M. oryzae* PO6-6 and compatible with *M. oryzae* KI215 (Figs. 3B, 3C, and 4). In the line with that, transgenic plants expressing *Pii-2* or *Pii-2Δ* in *Pi5-1* cDNA carrying background also exhibited the obvious resistance to *M. oryzae* PO6-6 and susceptibility to *M. oryzae* KI215 (Figs. 3B, 3C, and 4). These data indicate that *Pii* and *Pi5* genes function interchangeably in triggering ETI.

CRISPR/Cas9-mediated mutation of *Exo70F3* in *Pi5* transgenic rice

Exo70F3 was previously reported as an interactor of AVR-Pii and critical for *Pii*-mediated resistance. Loss of *OsExo70F3* by RNAi abolished *Pii*-mediated resistance (Fujisaki et al., 2015). Therefore, we hypothesized that knockout mutations of *OsExo70F3* would cause loss of *Pi5*-mediated resistance. In order to test this hypothesis, we generated OsExo70F3 loss-of-function mutants with the CRISPR/Cas9-mediated gene editing method in the transgenic *Dongjin* plants expressing *Pi5-1* and *Pi5-2* genomic DNAs described in Figure 3B. The resulting knockout mutants, *exo70f3-1*, *exo70f3-2* and *exo70f3-3*, carried distinct modifications in the coding region of *OsExo70F3* (Fig. 5A). Strikingly, a premature stop codon caused by the shift of reading frame in the coding region (*exo70f3-1* and *exo70f3-2*) and loss of a large fragment (*exo70f3-3*) (Supplementary Fig. S4) did not affect *Pi5*-mediated resistance, evidenced by restricted lesion development (Figs. 5B and 5C).

DISCUSSION

The requirement of Pi5-1 and Pi5-2 in *Pi5*-mediated resis-

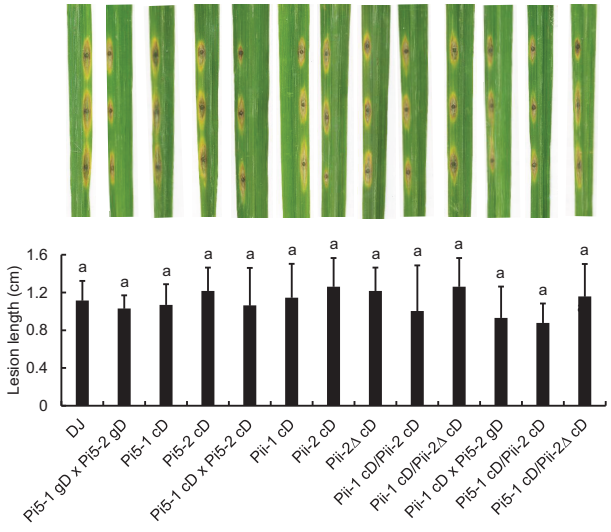

Fig. 4. Disease resistance of transgenic plants to *M. oryzae* KI215. Representative leaves showing disease lesions in six-week-old transgenic plants carrying indicated transgene(s) at nine dpi. DJ, *Dongjin* (susceptible cultivar); cD, cDNA; gD, genomic DNA. Lesion lengths analyzed by ANOVA ($\alpha = 0.05$), and Tukey's HSD test was used to conduct mean separation. The same letter indicates insignificant difference.

tance was validated by introducing both genomic DNA fragments to susceptible cultivar *Dongjin* (Lee et al., 2009). In order to dissect their functions, the protein coding sequences of both genes were validated. In this study, we generated transgenic plants expressing *Pi5-1* and *Pi5-2* cDNAs by crossing each of the overexpression lines. The obvious resistance to *M. oryzae* PO6-6 was demonstrated in transgenic lines expressing both cDNAs, indicating that they are sufficient to confer *Pi5*-mediated resistance.

The similarity of resistance specificity conferred by *Pii* and *Pi5* (Ebron et al., 2004; Selisana et al., 2017) led us to hypothesize that *Pii*-mediated resistance requires a pair of NLR genes that are allelic to those of *Pi5*. In this study, we provide several lines of evidence to prove the allelism between *Pii* and *Pi5*. Firstly, two counterpart genes, *Pii-1* and *Pii-2*, which share high similarity to *Pi5-1* and *Pi5-2*, respectively, were present in *Fujisaka5* carrying *Pii*. Secondly, the paired NLRs of Pii-1 and Pii-2 were necessary for *Pii*-mediated resistance as confirmed in RNAi plants of *Pii-1* and *Pii-2* (yielding susceptibility to *M. oryzae* PO6-6) and in transgenic plants coexpressing both *Pii-1* and *Pii-2* cDNAs (yielding resistance to). Lastly, the replacement of *Pii-1* by *Pi5-1* or *Pii-2* by *Pi5-2* retained an incompatible interaction to *M. oryzae* PO6-6.

Cell death is often an indicator of defense induced by ETI. Therefore, LM phenotypes have been studied in-depth to understand the molecular aspect of defense (Wu et al., 2008). Cell death phenotypes in tobacco are observed when expressing *PigmR*, whereas HR-like symptoms demand the

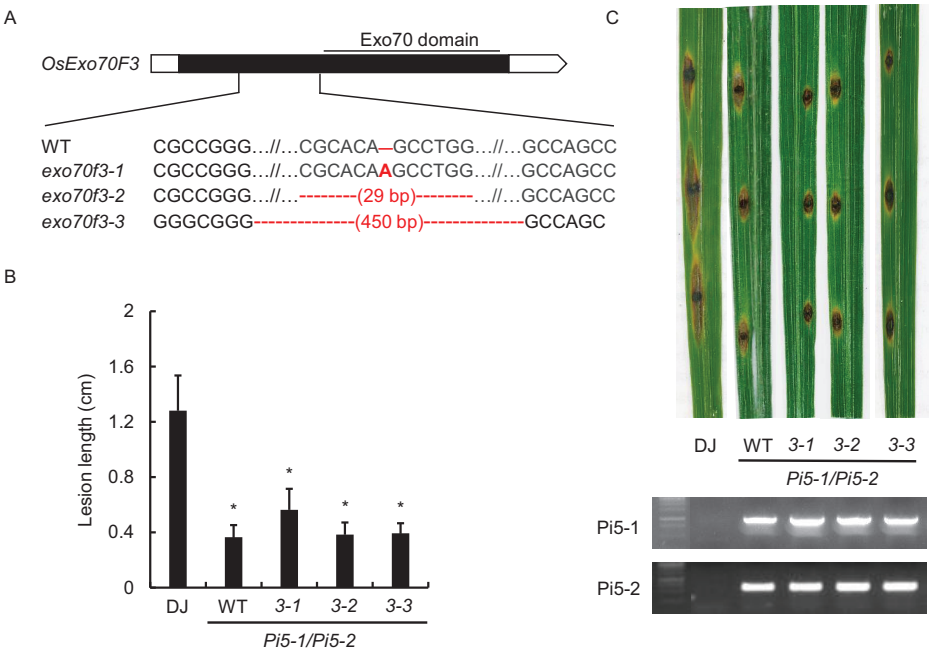

Fig. 5. Characterization of *Pi5* transgenic lines with *OsExo70F3* mutations. (A) Production of *OsExo70F3* loss-of-function mutants by CRISPR/Cas9 in transgenic rice cultivar *Dongjin* carrying *Pi5-1* and *Pi5-2*. Exon and untranslated regions are represented as black and white boxes, respectively. The Exo70 domain is indicated. Disease lesion lengths (B) and representative leaves (C) of the six-week-old mutant lines were measured at nine dpi with *M. oryzae* PO6-6. Asterisks represent statistical significance with Student's *t*-test, $P < 0.0001$. The presence of *Pi5* genes was validated by PCR using genomic DNA. DJ, *Dongjin* (susceptible cultivar); WT, transgenic rice carrying *Pi5-1* and *Pi5-2*; *3-1*, *exo70f3-1*; *3-2*, *exo70f3-2*; *3-3*, *exo70f3-3*.

presence of both AVR-Pia and Pia in rice (Deng et al., 2017; Satoh et al., 2014). Previously, the introduction of both *Pi5-1* and *Pi5-2* genomic DNAs into *Dongjin* did not cause an obvious morphological phenotype (Lee et al., 2009). In contrast, LM phenotype appeared in transgenic plants expressing both *Pi5* cDNAs, as well as transgenic plants expressing both *Pii* cDNAs, but was absent in single gene transgenic lines (Supplementary Figs. S2 and S3). A similar observation was made for the paired Arabidopsis NLRs, RPS4 and RRS1, where RPS4 auto-immunity depends partially on RRS1 (Heidrich et al., 2013). This suggests that overexpression of both paired NLR genes leads to constitutive defense activation that functions as a cell death trigger.

The sequence variation in NB-LRR genes is the result of a long co-evolution between rice and rice blast. Therefore, the significant difference in the last intron of *Pii-2* and the presence of longer *Pii-2* mRNA led us to investigate the function of *Pii-2* truncated form (*Pii-2*Δ*) which is absent in Fujisaka5* cDNA library. However, transgenic plants carrying either *Pii-2* or *Pii-2*Δ *additionally to Pii-1* could confer resistance to AVR-Pii and AVR-Pi5 carrying isolate PO6-6. The integration of an effector target into plant NLRs has been reported as a novel conserved mechanism called the integrated decoy model (Césari et al., 2014). RIN4 acts as molecular switch and contains two nitrate-induced (NOI) domains that function in suppressing PTI (Ray et al., 2019). In this regard, a conserved NOI core motif (PxFGxW) of the AvrRpt2-cleavage site was observed in the *Pi5-2* C-terminus (Nishimura et al., 2015) that is also retained in both *Pii-2* variants. Notably, a genome-wide association study using the rice diversity panel1 identified a new resistance allele at the *Pi5* locus of a resistance cultivar 301279 (Kang et al., 2016). The *Pi5-2* allelic gene newly identified was found to have a short C-terminus; therefore, lacking the NOI motif but retaining resistance function based on RNAi experiments. This suggests that the NOI motif of *Pii-2* and *Pi5-2* may not be critical for resistance, although further studies are required to confirm this.

The sequence similarity, resistance spectrum, and location on the chromosome raised the question about the identity of AVR-Pii and AVR-Pi5 effectors. AVR-Pii was previously isolated via an association genetics approach among 23 *M. oryzae* isolates (Yoshida et al., 2009). Subsequently, a comparative study of 77 *M. oryzae* isolates from the International Rice Research Institute (IRRI) has shown a correlation between the presence of AVR-Pii and activation of *Pi5*-mediated resistance, except for a few isolates that lack AVR-Pii but are avirulent on the *Pi5* line (Selisana et al., 2017), suggesting that AVR-Pii is recognized by the Pi5-1/Pi5-2 pair or that an additional unknown Avr effector (AVR-Pi5) is present. In a recent study, a considerable inconsistency was found between the presence of AVR-Pii sequence in a large collection of *M. oryzae* isolates and *Pii*-mediated resistance in rice (i.e., resistant but without AVR-Pii or susceptible with AVR-Pii) (Lu et al., 2019), implying that another unknown AVR-Pii effector is present or that an additional factor is required for AVR-Pii function. Since AVR-Pi5 has not been identified independently, we hypothesized that testing cross-functionality of *Pii* and *Pi5* alleles might be useful to determine if the *Pii* and *Pi5* genes have identical function. Accordingly, we generated the combination of *Pii-1* and *Pi5-2* along with *Pi5-1* and *Pii-2* to examine the cooperation of two genes. The crossed line of *Pii-1* cDNA-overexpressing plant and *Pi5-2* genomic DNA-carrying as well as transgenic plants expressing *Pi5-1* cDNA and *Pii-2* or *Pii-2*Δ were resistant to *M. oryzae* PO6-6 which possesses AVR-Pii and AVR-Pi5, suggesting that Pii and Pi5 components can function cooperatively in conferring disease resistance. However, whether the resistance is *Pii* or *Pi5*-mediated is unknown. Therefore, the six amino acid difference in Pii-1/Pi5-1 and eleven in Pii-2/Pi5-2 might not significantly alter Avr recognition. Nevertheless, if the AVR-Pi5 is distinct from AVR-Pii, the crosses will be useful to understand the interesting mechanism of Avr recognition of *Pii* and *Pi5*.

The Exo70 protein in the exocyst complex is important in tethering and fusion of the vesicles and plasma membrane at the site of polarized exocytosis (Munson and Novick, 2006). In plants, OsExo70F3 is known to interact with AVR-Pii and is critical for *Pii*-mediated resistance (Fujisaki et al., 2015). Therefore, in our study, the involvement of OsExo70F3 in Pi5 action was evaluated by examining susceptibility of *Exo70F3* mutations in the *Pi5* background. Interestingly, *Pi5*-mediated resistance does not require Exo70F3 whereas *Pii* does, illuminating a difference in defense mechanisms among two *R* genes at the same locus. Differential requirement of OsExo70F3 for *Pi5* and *Pii*-mediated resistance is puzzling because both NLR pairs seem to recognize the same Avr effector, AVR-Pii. One would hypothesize that AVR-Pii may target multiple Exo70 variants and an unidentified Exo70 variant might be required for Pi5. Alternatively, Pi5-1 and Pi5-2 may recognize an unknown Avr effector. However, so far, most of the analyzed *M. oryzae* isolates that are avirulent to *Pi5* carry AVR-Pii. It is unlikely, although not impossible, that Pi5-1 and Pi5-2 recognize an unknown Avr effector. Therefore, together with the identification of AVR-Pi5, functional verification of exocytosis components will pave the way to uncover the hidden mechanism of *Pi5*-resistance.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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