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Identification of an avirulence gene, a*vrxa5,* from the rice pathogen *Xanthomonas oryzae* pv. *oryzae*

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Xanthomonas oryzae pv. *oryzae*, the causal agent of bacterial blight in rice, interacts with rice plants in a gene-for-gene manner. The specificity of the interaction is dictated by avirulence (*avr*) genes in the pathogen and resistance (*R*) genes in the host. To date, no *avr* genes that correspond to recessive *R* genes have been isolated. We isolated an *avrBs3/pthA* family gene, *avrxa5*, from our previously isolated clone p58, which was originally from strain JXOIII. The *avrxa5* gene converted the PXO99^{A} strain from compatible to incompatible in rice cultivars containing the recessive *xa5* gene, but not in those containing the dominant *Xa5* gene. Sequencing indicated that *avrxa5*, which is highly similar to members of the *avrBs3/pthA* family, encodes a protein of 1238 amino acid residues with a conserved carboxy-terminal region containing three nuclear localization signals and a transcription activation domain. It has 19.5 34-amino-acid direct repeats, but the 13th amino acid is missing in the fifth and ninth repetitive units. Domain swapping of the repetitive regions between *avrxa5* and *avrXa7* changed the avirulence specificity of the genes in *xa5* and *Xa7* rice lines, respectively. This indicates that *avrxa5* is distinct from previously characterized *avrBs3/pthA* members. The specificity of *avrxa5* toward recessive *xa5* in rice could help us better understand the molecular mechanisms of plant–pathogen specific interactions.

rice bacterial blight, *Xanthomonas oryzae* pv. *oryzae*, *avrxa5*, avirulence, recessive resistance

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of rice bacterial blight, a disease that causes serious yield losses in much of Asia and parts of Africa [1–4]. The *Xoo*-rice pathosystem has been used as a model to understand the molecular interactions governed by the gene-for-gene principle between plants and pathogens [3,5]. The most effective way to control bacterial blight is to grow resistant (*R*) rice cultivars; however, disease resistance can be lost in resistant cultivars if they are grown for more than 3–5 years. This is because the pathogen generates new effector(s) in a new dominant race to overcome specific recognition by the matching *R* gene in plants [3]. Virulence differentiation of *Xoo* is thought to be mediated by different members of the *avrBs3/pthA* family in different strains or races. Genome sequences indicate that there are 15 members of the *avrBs3/pthA* family in strain KACC10311 [6], 17 in MAFF311018 [7] and 19 in PXO99^{A} [8]. AvrBs3/PthA proteins share unique structural features: highly conserved N- and C-termini, a leucine zipper (LZ), three nuclear localization signals (NLSs), and an acid transcriptional activation

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domain (AD) near the C terminus, nearly identical repeats of 34 amino acids in their central portions, and variable amino acids at the twelfth and thirteenth sites of the repeats [9–16]. The number and arrangement of the repeat units differ [9,16], contributing to virulence function and specificity during the elicitation of resistance and virulence in host cultivars without resistance genes [17,18]. Structure-function studies that have examined the domains of the AvrBs3/PthA family indicate that the AD, NLS, and repeat regions are important for avirulence and virulence specificity [19–25]. AvrBs3/PthA proteins are transcriptional activator-like (TAL) effectors, which are trafficked via a type-III secretion system [22,26,27] into the plant cell cytoplasm. NLSs are responsible for the translocation of these proteins into the nucleus, where they regulate the expression of genes required for genotype-specific hypersensitive responses (HR) or pathogenicity [15,21,23,28]. The HR involves rapid localized cell death associated with the arrest of pathogen ingress. It is led by a single pathogen *avr* gene, which is recognized only in a cultivar with a cognate dominant *R* gene. Thus, *avr* genes restrict the pathogen's host range, an effect that is deleterious to the pathogen and therefore unlikely to be the genes' primary function [9]. The best examples in *Xoo* are *avrXa7* [15,29], *avrXa10* [30,31] and *avrXa27* [32], which are recognized by the corresponding *Xa7*, *Xa10* and *Xa27* genes in rice, respectively; *Xa7* and *Xa10* have not yet been isolated. Currently, 35 resistance genes for bacterial blight have been identified in rice, and the full-length DNA sequences of nine (*Xa1* [33], *Xa3* [34], *Xa21* [35], *xa5* [36], *xa13* [38], *xa24* [25], *Xa21D* [38], *Xa26* [39], and *Xa27* [32]) have been isolated. However, the only interaction fully characterized to date is that between *avrXa27* and *Xa27* [32]. *Xa27* differs from its susceptible allele within the putative promoter region, and its expression is specifically induced by AvrXa27.

The above *R* genes are diverse in their predicted structures and subcellular localization. There is also little known about how a TAL effector in *Xoo* can modulate the expression of a recessive *R* gene, such as *xa5*, in rice. *xa5* is an allele of a gene on chromosome 5 that encodes the gamma subunit of the general transcription factor TFIIA (*TFIIAγ* or *Xa5*) [5,36]. TFIIAγ in the resistant allele differs from that in the susceptible allele by one codon, resulting in a substitution from valine to glutamic acid at residue 39. TFIIAγ stabilizes the binding of the TATA-box binding protein to the TATA box. It plays an important role in transcriptional activation, functioning either as an anti-repressor or a co-activator [5,40], but has not previously been associated with defense in animals or plants. Identifying such a TAL effector in *Xoo* that functions as a cognate gene to *xa5* will facilitate our understanding of the function of *xa5* in rice.

Previously, we isolated the cosmid p58 from a genomic DNA library of the strain JXOIII [41]. The transconjugant of PXO99^{A} with this cosmid induced a HR in the rice cultivar IRBB5, which contains the recessive *R* gene *xa5* [41]. This result prompted us to identify an *avr* gene that specifically matches the *xa5* gene in rice. Interestingly, the isolated *avr* gene belongs to the *avrBs3/pthA* family. The gene is herein designated *avrxa5,* and is distinct from other *avrBs3/pthA* family members based on its repeat organization.

1 Materials and methods

1.1 Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *Xoo* bacteria were grown in nutrient broth medium (0.5% yeast extract, 1% polypeptone, and 1% sucrose) at 28°C. The *Escherichia coli* strains were grown in LB medium at 37°C. Culture media were supplemented with antibiotics at the following concentrations (μg/L): rifampicin (Rif), 100; spectinomycin (Sp), 25; kanamycin (Km), 20; ampicillin (Ap), 50 when required.

1.2 Isolation of *avrBs3/pthA* genes from the cosmid p58

Originally, the cosmid p58 changed the PXO99^{A}–rice IRBB5 interaction from compatible to incompatible, indicating that an avirulence gene matching IRBB5 exists. To isolate this *avr* gene, p58 was first digested with *Bam*H I, producing five fragments. The fragments were collected individually from an agarose gel and then cloned into the cosmid pUFR034, giving rise to pB1, pB2, pB3, pB4 and pB5. A 359 bp *Eco*R I-*Bam*H I fragment that contained NLS motifs of an *avrXa3* gene [42] was used as a probe in Southern hybridization to identify which of the five plasmids contained *avrBs3/pthA* genes (Figure 1A). Probe labeling and hybridization were carried out using the DIG labeling kit (Roche) in accordance with the manufacturer's instructions. The plasmids containing *avrBs3/pthA* signals were confirmed by PCR with a pair of primers that cover the region of NLSs (F: 5′-TTAGGTACCGCATTGTTGCCCAGTTATCTCGC-3′; R: 5′-TAATCTAGAGCTGGGCGCATCAAGGTCACGCT-3′). Molecular protocols for PCR assays, plasmid DNA extraction, and DNA restriction enzyme digestion, ligation and transformation were performed as described previously [45]. Southern-hybridized *Bam*H I fragments in the positive plasmids (pB1 and pB4) were transferred into the vector pUAVPD at the *Bam*H I site. The ORF direction of *avrBs3/pthA* genes was correctly inlayed, with PCR confirmation using primers F (5′-CTCCAGGCATCAGGGATGAAAAGGG-3′) and R (5′-TGCCACTGCTGGATCATCTGCTGGA-3′), producing pUAVB1 and pUAVB4, respectively (Table 1).

1.3 Domain swapping of the repetitive regions between *avrxa5* and *avrXa7*

To exchange the repetitive region of *avrxa5* with the corre-

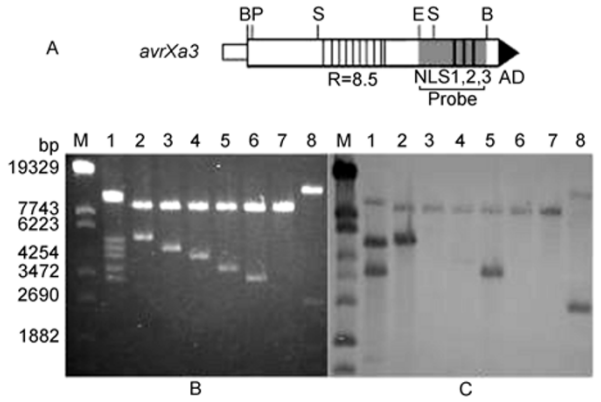

Figure 1 Isolation of *avrBs3/pthA* genes in the cosmid p58, which was originally from the strain JXOIII of *Xanthomonas oryzae* pv. *oryzae*. A, Structural features of *avrBs3/pthA* family members exemplified by the *avrXa3* gene [42]. Letters on the top stand for restriction enzyme site: B, *Bam*H I; P, *Pst* I; S, *Sph* I; E, *Eco*R I. R=8.5 means that the repeat number of the 102 bp repeat unit is 8.5. The *Eco*R I-*Bam*H I fragment from *avrXa3* was used as the probe (shaded region). B. Isolation of individual *Bam*H I fragments from the cosmid p58. Lane 1, five *Bam*H I-digested fragments from p58, approximately 5.1, 4.5, 4.2, 3.6 and 3.1 kb in size, were separately ligated into pUFR034, giving rise to pB1 (lane 2), pB2 (lane 3), pB3 (lane 4), pB4 (lane 5), and pB5 (lane 6). Lane 7, pUFR034 and lane 8, pUAV45K, used as negative and positive controls, respectively. C, Southern hybridization of the *avrBs3/pthA* genes in the cosmids from (B).

sponding fragment of *avrXa7*, the plasmids pB4 and pZWavrXa7 [29] were digested with *Bam*H I. The fragments containing the largest sections of *avrxa5* and *avrXa7* genes were transferred into the pBluescript vector at the *Bam*H I site, resulting in pBB4 and pBarvXa7, respectively. The new vectors were digested with *Sph* I, and gel-purified fragments of approximately 2.5 kb (*avrxa5*) and 3.1 kb (*avrXa7*) were replaced by each other in pBB4 and pBavrXa7, generating pBavrxa5-7 and pBavrXa7-5, respectively. The new constructs were confirmed by PCR with primers F (5′-TTAGGTACCGCATTGTTGCCCAGTTATCTCGC-3′) and R (5′-TAATCTAGAGCTGGGCGCATCAAGGTCACGCT-3′). The new constructs were digested with *Bam*H I, and the individual fragments, *avrxa5-7* and *avrXa7-5*, were ligated into pUAVPD to produce pUavrxa5-7 and pUavrXa7-5, respectively.

1.4 Pathogenicity tests in rice

Rice line IR24, which is susceptible to PXO99^{A} and JXOIII, was used for virulence assays. IRBB5 and IRBB7 contain resistance genes *xa5* and *Xa7* and were used for avirulence activity assays of *avrxa5* and *avrXa7*, respectively; the latter was used as a control. To exclude the possibility that lines IRBB5 and IR24 contained numerous other stretches of introgressed DNAs and additional *R* genes that may have recognized our avirulence gene, the following rice lines were used: Nipponbare (*japonica*), which contains the dominant allelic site of *xa5* gene [43]; NBB5, which is the cross of Nipponbare and IRBB5 (*indica*), and contains the recessive and homozygous *xa5* gene [43]; and NPT21, which contains the homozygous *xa5* gene [44]. The *avrBs3/pthA* gene clones were introduced into PXO99^{A} by electroporation. The transformants were cultured in NA medium for 36 h until the bacterial suspension reached a concentration of 3×10^{8} cfu/mL. Bacterial cells were first injected into 2-week-old rice leaves using needleless syringes. The water soaking symptoms were scored 3 d after injection. Adult rice plants were inoculated by leaf-clipping the last emerged leaves (10 leaves together) at the bolting stage. Lesion length was measured and scored 14 d after inoculation. The experiment was repeated three times with similar results.

1.5 Bacterial growth in planta

Bacterial cells were injected into young rice plant leaves as stated above. For each strain, a 0.5 cm^2 sample was collected from leaves with penetrated sites. The leaf was rinsed with sterile water, submerged in 75% alcohol for 30 min and then rinsed with sterile water three times. Sterilized samples were ground into powder in a 1.5-mL eppendorf tube. After 1 mL NB medium was added, the sample was kept stationary for 30 min at room temperature. The NB medium was serially diluted 10-fold, and 200 μL of the diluted solution was plated on NA plates containing suitable antibiotics. The bacterial colonies were counted after 3–4 d cultivation at 28°C. The assay was repeated four times with similar results.

1.6 Nucleotide sequencing and data analysis

To sequence the repetitive region of the *avrxa5* gene, which is inlayed in an *Sph* I fragment, we digested the pB4 clone with *Sph* I and cloned the fragment into the plasmid pMD 18-T, which was then named pMB4S. The EZ-Tn5™ transposon was inserted in the central *Sph* I fragment in pM4BS (EZ-Tn5 <R6Kgori/KAN-2> Insertion Kit, EPICENTRE® Biotechnologies), giving rise to pMB4STn5. The positive clone pMB4STn5 was sequenced twice using the M13-F, M13-R, KAN-2 FP-1 forward primer and R6KAN-2 RP-1 reverse primer, as indicated in the kit instructions. DNA sequencing was performed at Jinsite Corporation (China). The sequence data were analyzed with the BLAST program at the National Center for Biotechnology Institute and by Vector NTI software (Blue Sky Software Corp.). The sequence for 500 bp upstream and 300 bp downstream of the *avrxa5* gene in the clone p58 was obtained in each direction after the *Bam*H I fragment was sequenced. The coding sequence of amino acids 12 and 13 in each repeat unit of the repetitive regions was aligned with those in the repetitive regions of other representatives of the *avrBs3/pthA* family in *Xoo* using Vector NTI software. The nucleotide sequence is in the GenBank database; the whole *avrxa5* gene has been assigned the accession number FJ593881.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Property	Resource
E. coli		
DH5α	*Φ901acZΔm15*, *recA1*	Our lab
X.oryzae.pv.*oryzae*		
JXOIII	Rif ^{r}, wild type, Japanese race 3	Our lab
PXO99^A	Rif ^{r}, wild type, Philippine race 6	Our lab
PXO99^A (pUFR034)	Rif ^{r}, Km^r, wild type with a empty vector	Our lab
PXO99^A (pUAVPD)	Rif ^{r}, Km^r, wild type with a universal complementary vector	Our lab
PXO99^A (p58)	Rif ^{r}, Km^r, PXO99^A strain containing p58 cosmid	This study
PXO99^A (pUAVB1)	Rif ^{r}, Km^r, PXO99^A strain containing pUAVB1 cosmid	This study
PXO99^A (pUAVB4)	Rif ^{r}, Km^r, PXO99^A strain containing pUAVB4 cosmid	This study
PXO99^A (pZWavrXa7)	Rif ^{r}, Sp^r, PXO99^A strain containing pZWavrXa7 cosmid	This study
PXO99^A (pUavrxa5-7)	Rif ^{r}, Km^r, PXO99^A strain containing pUavrxa5-7 cosmid	This study
PXO99^A (pUavrXa7-5)	Rif ^{r}, Km^r, PXO99^A strain containing pUavrXa7-5 cosmid	This study
Plasmid		
pAVRprobe	Km^r, a 359 bp *Eco*R I-*Bam*H I fragment of *avrXa3* gene ligated in pBluescript KS II (–) for Southern blot as a probe.	[41]
pBluescript KSII(–)	Ap^r, 3.0 kb,Phagemid, pUC derivative,	Our lab
pK18mobGII	Km^r, 5.9 kb, pUC derivative, pUC18 polylinker, *mob*, *oriV*	[49]
pUAV45K	Km^r, a 5.8 kb *Kpn* I fragment containing *avrXa3* gene in pUFR034	[42]
pUAVPD	Km^r, a derivative of pUAV45K used as a universal complementary vector containing the upper and down regions without the *Bam*H I fragment of the *avrXa3* gene.	[41]
p034B54S	Km^r, a 2.4 kb *Bam*H I fragment of *avrBs3/pthA* gene in pUFR034	Our lab
pUFR034	Km^r, 8.7 kb, *IncW*, *Mob(p)*, *Mob*$^+$, *LacZα+*	[50]
pUFR034(B)	Km^r, pUFR034 with *Bam*H I end filled in	Our lab
pZWavrXa7	Sp^r, *avrXa7* gene cloned in pHM1 mediated by pBluescript II KS$^+$	[15]
p58	Km^r, *avrBs3/pth*A positive clone from JXOIII genomic DNA library in pUFR034	[41]
pB1	Km^r, a 5.3 kb *Bam*H I fragment derived from p58 into pUFR034	This study
pB2	Km^r, a 4.5 kb *Bam*H I fragment derived from p58 into pUFR034	This study
pB3	Km^r, a 4.1 kb *Bam*H I fragment derived from p58 into pUFR034	This study
pB4	Km^r, a.3.7 kb *Bam*H I fragment of *avrxa5* from p58 into pUFR034	This study
pB5	Km^r, a 2.4 kb *Bam*H I fragment derived from p58 into pUFR034	This study
pUAVB1	Km^r, a 5.3 kb *Bam*HI fragment of *avrBs3/pthA* gene derived from p58 into pUAVPD	This study
pUAVB4	Km^r, a 3.7 kb *Bam*H I fragment of *avrxa5* from p58 into pUAVPD	This study
pBB4	Ap^r, a 3.7 kb *Bam*H I fragment of *avrxa5* gene into pBluescript II SK (-)	This study
pBarvXa7	Ap^r, a 4.2 kb *Bam*H I fragment of *avrXa7* gene into pBluescript II SK (-)	This study
pUavrxa5-7	Ap^r, a 3.1 kb *Sph* I fragment of *avrXa7*gene substituting the corresponding *Sph* I fragment of *avrxa5* in pUAVPD	This study
pUavrXa7-5	Ap^r, a 2.5 kb *Sph* I fragment of *avrxa5* gene substituting the corresponding *Sph* I fragment of *avrXa7* in pUAVPD	This study
pKB4	Km^r, a 3.7 kb *Bam*H I fragment of *avrxa5* gene into pK18mobGII	This study
pMB4S	Ap^r, a 2.5 Kb *Sph* I fragment of *avrxa5* gene into pMD 18-T	This study
pMB4STn5	Ap^r, Km^r, Tn5 inserted in the central *Sph* I fragment in pM4B4S	This study

2 Results

2.1 Isolation of two *avrBs3/pthA* genes from the cosmid p58

To determine whether the cosmid p58, which was isolated from the genome library of the strain JXOIII of *Xoo* [41], contains any *avrBs3/pthA* family genes, we conducted Southern hybridization using a conserved 359 bp *Eco*R I-*Bam*H I fragment from *avrXa3* [42] as a probe (Figure 1A). Two *Bam*H I cosmid fragments hybridized to the probe, even though there were five *Bam*H I-digested bands (Figure 1B), suggesting that the cosmid contains two *avrBs3/pthA* genes (Figure 1C). To isolate them, we digested the cosmid DNA with *Bam*H I and then placed five individual fragments into pUFR034, giving rise to pB1, pB2, pB3, pB4, and pB5 (Figure 1B). Southern hybridization with the same probe verified that plasmids pB1 and pB4 carried a 5.3 kb and a 3.7 kb *avrBs3/pthA* fragment, respectively (Figure 1C). Due to the highly conserved *Bam*H I sites at the 5′ and 3′-termini

of the *avrBs3/pthA* family (Figure 1A), we conclude that plasmids pB1 and pB4 each contain a single different *avrBs3/pthA* gene.

2.2 *avrxa5*, an *xa5*-matching gene in JXOIII strain of *X. oryzae* pv. *oryzae*

We isolated each *Bam*H I fragment from the selected clones to examine the spectrum of *avr* and *R* gene interactions. We found that the promoter regions, and the remaining region after the stop codons of *avrBs3/pthA* genes, are almost the same in *Xoo* (data not shown). After we confirmed whether the isolated *Bam*H I fragments were inlayed correctly between the *avrBs3/pthA* highly conserved promoter and the 3′ region of the *Bam*H I site in the vector pUAVPD (a universal vector for complementation of *avrBs3* family members to PXO99^{A} strain [41]), pUAVB1 and pUAVB4 were transformed into strain PXO99^{A}. We injected the strains into rice seedlings and found that p58 and pUAVB4 altered the PXO99^{A}–IRBB5 interaction from compatible to incompatible (the injected area became brown without water-soaking symptoms appearing), but they did not convert compatible interactions in PXO99^{A}–IR24 (Figure 2A). The browning reaction is the typical HR phenotype triggered by the strain JXOIII (Figure 2A). By contrast, the recipient PXO99^{A} and the other transconjugants of PXO99^{A} (with either pUAVB1 or pUAV45K) did not trigger the HR in rice IRBB5 (Figure 2A). These results demonstrate that the *avrBs3/pthA* gene contained in pUAVB4 is an avirulence gene.

The transconjugant strains were also tested for growth in rice IRBB5, and showed similar trends in the assay. Importantly, the transconjugant of PXO99^{A} with pUAVB4, like p58, grew significantly less than the recipient itself in IRBB5, similar to the growth of strain JXOIII (Figure 2B). This indicates that the *avrBs3/pthA* gene in pUAVB4, originally from p58, which was in turn originally from strain JXOIII, contributed to avirulent function in rice IRBB5.

The effect of the genes on avirulence and virulence was qualified using lesion length measurements after leaf-clip inoculation of IRBB5 and IR24, respectively. Avirulence activity was measured using the fully virulent strain PXO99^{A} containing each of two Southern-hybridized *Bam*HI fragments of p58. To distinguish the effects of resistance from loss of virulence, *avrXa7* was used as a control. The *avrBs3/pthA* gene contained in either pUAVB4 or p58 significantly reduced lesion length, to less than 0.5 cm as long as lesions caused by the strain JXOIII (Figure 3A). Another *avrBs3/pthA* gene (5.3 kb *Bam*H I fragment) in pUAVB1, or *avrXa7* as a control, had little effect on the length of lesions when the recipient PXO99^{A} was inoculated onto IRBB5 (Figure 3A). The clip edges inoculated with strain JXOIII or with the transconjugants of PXO99^{A} (with either p58 or pUAVB4) were brown, with few developed

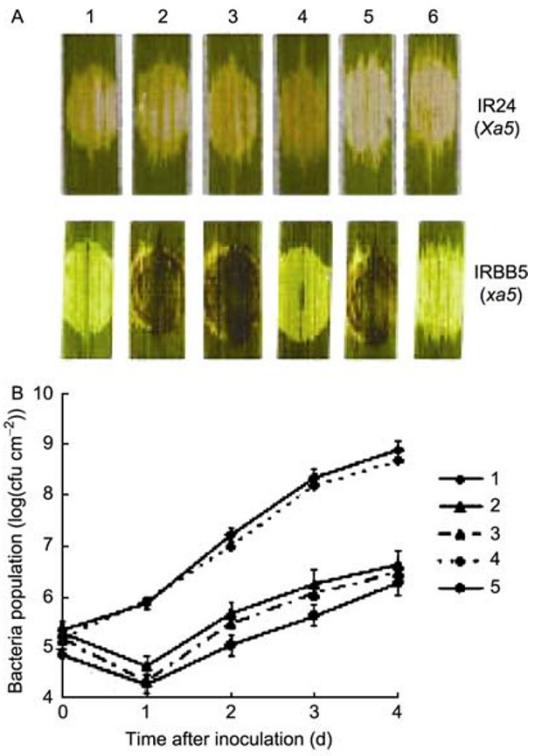

Figure 2 Virulence phenotype of *avrxa5* gene of *Xanthomonas oryzae* pv. *oryzae* on rice. A, Each strain was inoculated using a needleless syringe onto 2-week-old rice seedlings, IR24 (*Xa5*) and IRBB5 (*xa5*). Leaves were photographed 3 d after inoculation. 1, PXO99^{A} (pUFR034); 2, JXOIII; 3, PXO99^{A} (p58); 4, PXO99^{A} (pUAVB1); 5, PXO99^{A} (pUAVB4); 6, PXO99^{A} (pUAVPD). B, Populations of PXO99^{A} with *avrxa5* or derivatives in leaves of IRBB5. Curves represent the population of bacteria from the following strains: 1, PXO99^{A} (pUAVPD); 2, JXOIII; 3, PXO99^{A} (p58); 4, PXO99^{A} (pUAVB1); 5, PXO99^{A} (pUAVB4). Populations are expressed as CFU/cm^{2} of infected leaves. Error bars indicate±SD.

symptoms (Figure 2A). By contrast, the strain PXO99^{A} (either containing *avrBs3/pthA* in pUAVB1 or pUAVB4, or *avrXa7*) and the strain JXOIII caused normal bacterial blight, as per the wild type strain PXO99^{A} in IR24, which has an *Xa5* gene (Figure 3B). These results demonstrate that the *avrBs3/pthA* gene in pUAVB4 is an *avr* gene that matches *xa5* in rice IRBB5. It is herein designated as *avrxa5*.

2.3 Replacing the repetitive region of *avrxa5* with *avrXa7* alters avirulence specificity in rice

We replaced the central *Sph* I fragment of *avrxa5* in pUAVB4 with the central *Sph* I fragment of *avrXa7*, giving rise to *avrxa5-7*; this ensured that the associated phenotypes were attributable to regions within the *Sph* I fragment (Figure 4A). As a control, the repetitive region within the *Sph* I fragment of *avrXa7* was exchanged with the *Sph* I fragment of *avrxa5*, resulting in *avrXa7-5* (Figure 4B). These clones, in turn, were cloned into the universal vector pUAVPD and introduced into PXO99^{A}. PXO99^{A} with the parental gene

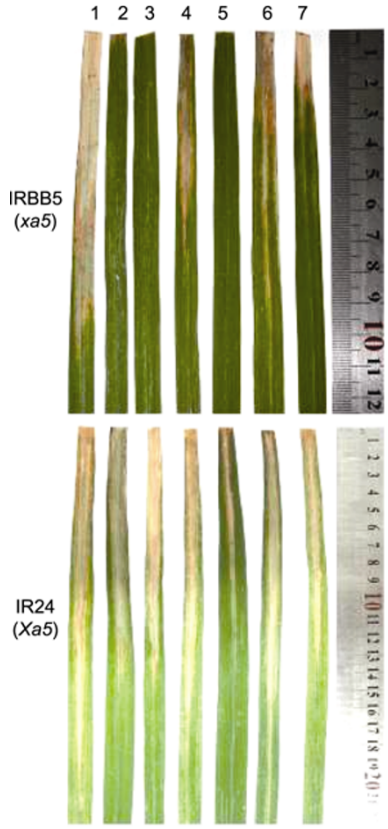

Figure 3 Avirulence and virulence activity of the *avrxa5* gene of *X. oryzae* pv. *oryzae* on adult rice plants. Each strain was inoculated by leaf clip onto rice IRBB5 (*xa5*) and IR24 (*Xa5*). Transformant PXO99^{A} (*avrxa5*) was avirulent and converted a compatible interaction with IRBB5 to an incompatible one with IR24. 1, PXO99^{A}; 2, JXOIII; 3, PXO99^{A} (p58); 4, PXO99^{A} (pUAVB1); 5, PXO99^{A} (pUAVB4); 6, PXO99^{A} (pUAVPD); 7, PXO99^{A} (pZWavrXa7) as a control.

avrXa7 elicited a resistant response on IRBB7 (Figure 4C), while the same strain with *avrXa7-5* was compatible on IRBB7 (Figure 4C), but incompatible on IRBB5 (Figure 4D). PXO99^{A} with the parental gene *avrxa5* produced a virulent response on IRBB7 (Figure 4C) and a resistant response on IRBB5 (Figure 4D), but PXO99^{A} with *avrxa5-7* triggered a resistant response on IRBB7 (Figure 4C) and was compatible on IRBB5 (Figure 4D). This indicates that the phenotypes were due to the exchange of the repetitive domains within the genes.

2.4 *avrxa5* triggers resistance response on other cultivars containing *xa5* gene

The reliance on isogenic lines IRBB5 and IR24 is insufficient to unequivocally determine that *avrxa5* corresponds specifically to *xa5*. We therefore inoculated the transconjugant of PXO99^{A} with *avrxa5* into rice lines Nipponbare (*Xa5*), NBB5 (*xa5*), and NPT21 (*xa5*). Nipponbare (*Xa5*) was susceptible to the strain infection, whereas NBB5 (*xa5*) and NPT21 (*xa5*) were incompatible (Figure 5). The clip edges inoculated with the transconjugant of PXO99^{A} with our *avrxa5* gene turned brown with few developed symptoms when the pathogen-rice interaction was incompatible (Figure 5), consistent with the symptoms in IRBB5. These genotypes of pathogen–rice interactions strongly suggest that the *avrxa5* gene contained in PXO99^{A} is recognized by *xa5*.

2.5 *avrxa5* is distinct from other *avrBs3/pthA* family members

Sequencing indicated that *avrxa5* (FJ593881) is 3717 bp in size and encodes a 1238 amino acid protein. The 102 bp repeat unit in *avrxa5* repeats 19.5 times (Figure 6A). Apart from this, the gene, including the promoter region, is as the same as those of *avrBs3/pthA* family genes in *Xoo* (data not shown). After the repetitive region, the putative protein has an LZ, three NLSs (1, 2, 3) and an AD domain at the C-terminal region (Figure 6A). Intriguingly, half of the amino acids at the12th and 13th sites of each repeat unit are histidine and aspartic acid (HD), and the 13th acid at the fifth and ninth repeat units is missing (Figure 6B). This suggests that the coding sequence represents the novel *xa5*-matching gene reported in the *avrBs3/pthA* family.

To understand the avirulence dynamics of different *avrBs3/pthA* members in *Xoo*, amino acids 12 and 13 in the repetitive regions coded by near-identical 102 bp direct repeats in *avrxa5* were aligned with other AvrBs3/PthA family members of *Xoo* that have precise functions in rice as showed in Figure 6. We found that the repetitive region for HDNG (G can be N, I, or S) amino acids may form the basic building block for development of different AvrBs3/PthA family proteins (Figure 6B). The fourth acid of this HDNG block can be missed without affecting the function. Moreover, conserved residues NING, NIHG, or NIH* (* residue missed) are found at the beginning of repetitive regions in some AvrBs3/PthA members, such as AvrXa10, PthXo7, PthXo6, and AvrXa7 (Figure 6B). The block and the conserved residues can be traced elsewhere along the sequences of these AvrBs3/PthA family members. This indicates that a new *avrBs3/pthA* gene in the pathogen would be generated by genetically adding certain repeat regions under the selection pressure of *R* genes in rice.

3 Discussion

We have demonstrated that the specificity for the avirulence activity of *avrxa5*, a novel member of the *avrBs3/pthA* family, depends on the unique structural features of the products of this gene family. The specificity for *xa5*-dependent avirulence activity on the rice cultivars IRBB5, NBB5, and NPT21 depends on the repetitive domain. The avirulence properties of the *avrBs3/pthA* family have been demonstrated by the identification of *avrXa7* [15,29], *avrXa10*

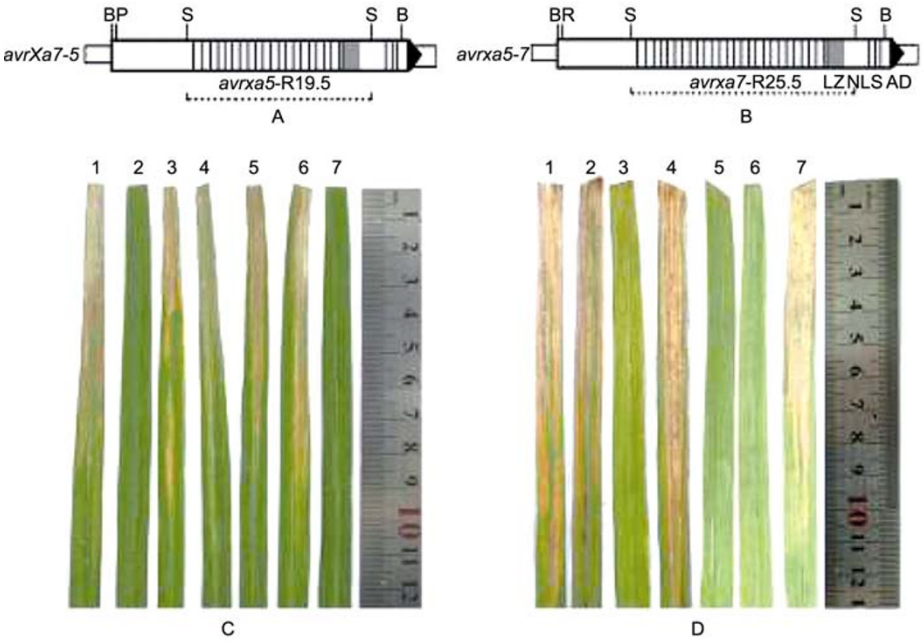

Figure 4 Avirulence and virulence activities of *avrxa5-7* and *avrXa7-5*, which were recombinations of the *avrxa5* repetitive region with the corresponding fragment from *avrXa7*. A and B, Schematic maps showing the recombination of *avrxa5* with *avrXa7* (A) and *avrXa7* with *avrxa5* (B). *avrxa5-7* was the replacement of *avrxa5* repetitive region (*Sph* I fragment) with the corresponding region from *avrXa7*; *avrXa7-5* was the *avrXa7* repetitive region (*Sph* I fragment) replaced by the corresponding region from *avrxa5* (*avrxa7-5*). Letters on the top of the schematic maps stand for restriction enzyme sites. B, *Bam*H I; P, *Pst* I; S, *Sph* I. R19.5 and R25.5 mean that the repeat numbers of the 102 bp repeat unit are 19.5 and 25.5, respectively. C and D, The effect of recombined *avrXa7-5* and *avrxa5-7* on inoculation phenotypes of resistant rice IRBB7 (C) and IRBB5 (D), respectively. Strains were inoculated by leaf clip onto IRBB7 and IRBB5. 1, PXO99^{A} (pUAVPD); 2, PXO99^{A} (pZWavrXa7); 3, PXO99^{A} (p58); 4, PXO99^{A} (pUAVB1); 5, PXO99^{A} (pUAVB4); 6, PXO99^{A} (pUavrXa7-5); 7,PXO99^{A} (pUavrxa5-7).

Figure 5 Avirulence specificity of the *avrxa5* gene of *X. oryzae* pv. *oryzae* on other rice alleles of *xa5*. The strain PXO99^{A} (*avrxa5*) was inoculated by leaf clip onto Nipponbare (*Xa5*), NBB5 (*xa5*) and NPT21 (*xa5*). Leaves were photographed 14 d after inoculation.

[30,31] and *avrXa27* [32]. These three genes have repeat domains of 25.5, 15.5, and 16.5 units, respectively, and a unique repeat domain defined by the 12th and 13th positions of each repeat unit. Replacing the repeat domain of *avrxa5* with that of *avrXa7*, which has avirulence specificity on the rice cultivar IRBB7, resulted in loss of avirulence specificity on the cultivar IRBB5. In contrast, *avrXa7* did not trigger a specific HR in IRBB5 when it was transferred into the recipient PXO99^{A} (Figure 4D), but the replacement of the repetitive region of *avrXa7* with that of *avrxa5* switched *avrXa7* specificity from IRBB7 (*Xa7*) to IRBB5 (*xa5*).

The HR genes of this family are presumably under selective pressure to maintain their virulence function, while avoiding recognition by the host defense system [15,38]. The adaptive process is presumably reflected in the variety of repeat domains in different genes of the family both from different strains and within strains [14,17,20,22,46]. *avrxa5*, originally from strain JXOIII, reflects its absence in PXO99^{A}, as PXO99^{A} lacks *avrXa7* and *avrXa10*, which are originally from strain PXO86. Although different TAL effectors within a given strain have qualitatively different effects [27,47], the results do not preclude cooperation between effectors. Given that AvrBs3/PthA proteins act as TAL effectors through type-III secretion system of the pathogen, it makes sense that Avrxa5 results in effector-triggered immunity in rice.

The length of a given pathogenicity gene may reflect the configuration of the variable regions necessary for virulence,

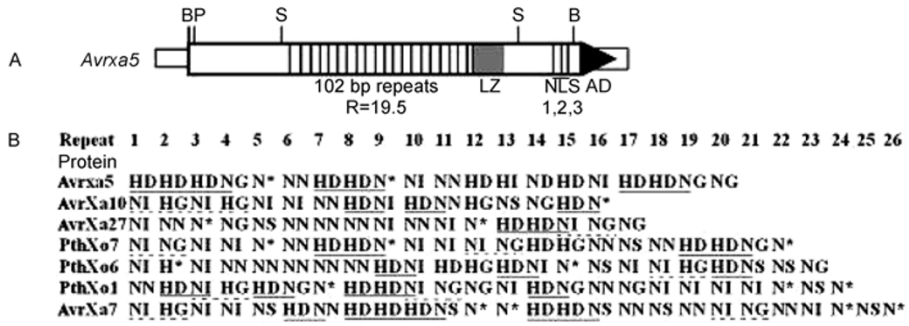

Figure 6 Alignment of the repetitive regions of function-identified AvrBs3/PthA proteins of *Xanthomonas oryzae* pv. *Oryzae*, represented by amino acids 12 and 13 of each repeat by using Vector NTI alignment software. A, Schematic map of Avrxa5 protein. See notes in Figure 1 about illustration. B, Avrxa5 has 19.5 repeats in the repetitive region and the 13th amino acid is missing within repeat 5 and 9. Apart from the different numbers of the 34 amino acid repeat unit in the AvrBs3/PthA family, amino acids 12 and 13 in the repeat unit play key roles in host specificity. The single amino acid code was used for each residue. Names of AvrBs3/PthA proteins in the GenBank database are listed on the left. The HDN residues underlined are the conserved core of all members listed. The NING or NIHG residues with broken underlines are at the beginning of the repetitive regions in some Avr/pth proteins. GenBank accession numbers. for proteins are: Avrxa5, AvrXa10, AvrXa27, PthXo7, PthXo6, PthXo1, and AvrXa7. Asterisks indicate positions of missing nucleotides or amino acid residues of the prototypic repeat unit.

which are arrived at by recombination and mutation. Strains of *Xoo* contain an estimated 15–20 copies of the *avrBs3/pthA* genes [3,6–8,41], which may provide ample substrate for generation of new repeat combinations. How the particular repeat domains mediate specificity for virulence or avirulence remains a mystery. We previously assumed that a new *avrBs3/pthA* gene arises from the combination of two *avrBs3/pthA* genes when the pathogen is under selective pressure from an *R* gene in rice [41]. Generally, the repeat number of the 34-amino-acid/102-bp repeat in the *avrBs3/pthA* gene determines its virulence or avirulence function, and the consistent variation at codons 12 and 13 in the individual repeats determines host specificity or adaptation to virulence [15,19,28,46]. Under selection pressure by host *R* genes, it is possible that the evolution of the 102-bp repeat unit is related to the mechanisms of virulence evolution in *Xoo*. Here, we found traces of the conserved residues (NIHG) and the basic building block (HDNG) at the 12th and 13th sites in the repeat regions of the AvrBs3/PthA family when they are collectively taken as a single sequence. This is consistent with two small *avrBs3/pthA* members in the strain JXOIII, which have only 1.5 repeats of the 34-amino-acid units [41].

Originally, an *avrxa5*-like gene isolated by Leach [48] was thought to interact with the *xa5* resistance gene in rice. However, no further evidence supported this until we identified the *avrxa5* gene in this study. The *xa5* gene encodes the gamma subunit of transcription factor IIA (TFIIAγ); it is recessive and does not conform to one of the typical resistance gene structural classes. TFIIAγ in resistant IRBB5 and susceptible IR24 differs in only two nucleotide substitutions, resulting in a single amino acid change [5,36]. However, this change is not in the promoter region, where, based on the binding specificity between TAL effectors and *R* gene promoter regions in plants [51,52], there should be an element bound by the 12th and 13th amino acids of the Avrxa5 repetitive region. Intriguingly, the expression patterns of *TFIIAγ* in susceptible IR24 and resistant IRBB5 rice are the same when the two lines are inoculated with strain PXO86 of *Xoo* [5]. Our *avrxa5* gene triggered HR resistance in xa5-containing IRBB5, NBB5 and NPT21, but not in IR24, Nipponbare, and other IRBB lines (data not shown). This suggests that TFIIAγ functions both as a general transcriptional factor and as *xa5*. Replacement of the *avrxa5* repetitive region with that from *avrXa7* resulted in a loss of avirulence specificity in IRBB5. Our hypothesis is supported by evidence that expression of a bZIP transcription factor gene (*OsTFX1*) and a gene located on chromosome 1 encoding the other small subunit of transcription factor IIA, *OsTFIIAγ1*, depends on the novel TAL effectors PthXo6 and PthXo7 in strain PXO99^{A} [46]. In the classical view, strain PXO86 is considered incompatible because of the presence of an avirulence protein, Avrxa5 [4]; but we presume that Avrxa5 is present in JXOIII, not in PXO86. Future experiments will be conducted to distinguish between these possibilities.

4 Conclusion

Here, we demonstrated that in addition to affecting the specific recognition of *xa5*, the pathogen alters avirulence via the Avrxa5 effector, which is highly similar within the same family but differs in the central repetitive region. *avrxa5* specificity occurred in resistant cultivars IRBB5, NBB5, and NPT21, but not in susceptible cultivars IR24, Nipponbare, and other IRBB lines. The avirulence to *xa5* can be altered by replacing its repetitive region with that of *avrXa7*. Thus, *avrxa5* from *Xoo* may facilitate our understanding of how specific recognition occurs when the major *R* gene in

rice is recessive, rather than dominant.

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