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Ortholog Alleles at *Xa3/Xa26* Locus Confer Conserved Race-Specific Resistance against *Xanthomonas oryzae* in Rice

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ABSTRACT **The rice disease resistance (*R*) gene *Xa3/Xa26* (having also been named *Xa3* and *Xa26*) against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes bacterial blight disease, belongs to a multiple gene family clustered in chromosome 11 and is from an AA genome rice cultivar (*Oryza sativa* L.). This family encodes leucine-rich repeat (LRR) receptor kinase-type proteins. Here, we show that the orthologs (alleles) of *Xa3/Xa26*, *Xa3/Xa26-2*, and *Xa3/Xa26-3*, from wild *Oryza* species *O. officinalis* (CC genome) and *O. minuta* (BBCC genome), respectively, were also *R* genes against *Xoo*. *Xa3/Xa26-2* and *Xa3/Xa26-3* conferred resistance to 16 of the 18 *Xoo* strains examined. Comparative sequence analysis of the *Xa3/Xa26* families in the two wild *Oryza* species showed that *Xa3/Xa26-3* appeared to have originated from the CC genome of *O. minuta*. The predicted proteins encoded by *Xa3/Xa26*, *Xa3/Xa26-2*, and *Xa3/Xa26-3* share 91–99% sequence identity and 94–99% sequence similarity. Transgenic plants carrying a single copy of *Xa3/Xa26*, *Xa3/Xa26-2*, or *Xa3/Xa26-3*, in the same genetic background, showed a similar resistance spectrum to a set of *Xoo* strains, although plants carrying *Xa3/Xa26-2* or *Xa3/Xa26-3* showed lower resistance levels than the plants carrying *Xa3/Xa26*. These results suggest that the *Xa3/Xa26* locus predates the speciation of A and C genome, which is approximately 7.5 million years ago. Thus, the resistance specificity of this locus has been conserved for a long time.**

Key words: **Bacterial blight; broad-spectrum resistance; durable resistance; *Oryza officinalis*; *Oryza minuta*; *Oryza sativa*.**

INTRODUCTION

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the major diseases affecting rice production. Breeding rice with the quality of broad-spectrum and durable disease resistance is one of the principal goals of rice improvement (Yang et al., 2008; Kou and Wang, 2010). Exploitation and utilization of major resistance (*R*) genes is an effective way to control bacterial blight. Wild germplasm is always recognized as an important repository of useful allelic variation for crop improvement including resistance against *Xoo*.

Harlan and de Wet (1971) proposed a concept involving three levels of the gene pools for classifying crop species and their wild relatives, according to the crossability between them. The genus *Oryza* includes two cultivated rice species, Asian cultivated rice *Oryza sativa* (AA genome) and African cultivated rice *Oryza glaberrima* (AA genome), and 22 wild species, representing AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ, and KKLL genomes (Ge et al., 1999; Lu et al., 2009; Ammiraju et al., 2010). According to the crossability between *O. sativa* and other *Oryza* species, the wild species also have been categorized as the primary, secondary, and tertiary gene pools for the cultivars (Khush, 1997). All AA genome *Oryza* species are relatively easy to cross with *O. sativa* and are regarded as the primary gene pool, the wild species with BB, CC, BBCC, CCDD, EE, and FF genomes constitute the secondary gene pool, and the remaining wild species with the GG, HHJJ, and KKLL genomes belong to the tertiary gene pool (Khush, 1997; Lu et al., 2009).

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The classic example of transfer of a disease resistance gene against *Xoo* from a wild species into cultivated rice is the introgression of *Xa21* from the AA genome wild rice *Oryza longistaminata* into Asian cultivated rice (Khush et al., 1990). *Xa21* has a broad-spectrum of resistance to nearly all races of *Xoo* in the Philippines (Cottyn and Mew, 2004); however, the activity of *Xa21* is developmentally regulated (Century et al., 1999; Zhao et al., 2009). Another broad-spectrum bacterial blight resistance gene *Xa23*, from *Oryza rufipogon* (AA), was transferred into Asian cultivated rice and is active throughout the lifecycle of the plant (Zhang et al., 2000). Both *Xa21* and *Xa23* are located on chromosome 11 in cultivated rice (Song et al., 1995; Zhang et al., 2000). Wild *Oryza* species belonging to the secondary gene pool have also provided valuable genes that confer resistance to *Xoo*, such as *Xa27* from *Oryza minuta* (BBCC) (Amante-Bordeos et al., 1992) and *Xa29(t)* from *Oryza officinalis* (CC) (Tan et al., 2004). The introgressed *Xa27* and *Xa29(t)* genes are located on chromosomes 6 and 1 in cultivated rice, respectively (Gu et al., 2004; Tan et al., 2004). However, only limited gene transfer is possible from the secondary gene pool into the Asian cultivated rice by crossing, because there is limited homology between *O. sativa* and these wild *Oryza* species (Khush, 1997).

Rice *Xa3/Xa26*, encoding a leucine-rich repeat (LRR) receptor kinase-type protein, is an *R* gene conferring resistance against *Xoo* (Sun et al., 2004; Xiang et al., 2006). Asian-cultivated rice consists of two major subspecies, *indica* (*O. sativa* L. ssp. *indica*) and *japonica* (*O. sativa* L. ssp. *japonica*). *Xa3/Xa26* was first identified in the *indica* rice cultivar Minghui 63 and named *Xa26* (Yang et al., 2003). Further study revealed that *Xa3*, an *R* gene conferring resistance against *Xoo*, and *Xa26* are actually the same gene and then it was renamed *Xa3/Xa26* (Xiang et al., 2006). *Xa3/Xa26* has been an important resistance gene for both *indica* and *japonica* rice production in China for a long time (Xu et al., 2004; Gao et al., 2010). Quantitative trail locus for disease resistance and defenses-responsive genes functioning and putatively functioning downstream of Xa3/Xa26 in defense signaling have been identified (Hu et al., 2008; Qiu et al., 2008, 2009; Kou et al., 2010). In this study, we used an ortholog (allele) mining strategy to isolate new *R* genes against *Xoo* in the *Xa3/Xa26* family from wild *Oryza* species. The *Xa3/Xa26* orthologs or alleles, *Xa3/Xa26-2* from *O. officinalis*, and *Xa3/Xa26-3* from *O. minuta* that encodes proteins different from Xa3/Xa26 protein can mediate a similar spectrum of resistance against *Xoo*. The *Xa3/Xa26*-carrying cultivars have been widely used in rice production for a long period of time. These results suggest that the *Xa3/Xa26* locus may confer a durable resistance.

RESULTS

Isolation of *Xa3/Xa26* Family Genes from *O. officinalis* and *O. minuta*

A total of nine and seven positive bacterial artificial chromosome (BAC) clones were identified after screening high-density hybridization filters from the *O. officinalis* and *O. minuta* BAC libraries, respectively, using a set of *Xa3/Xa26* family DNA probes. Based on the finger printing analysis of these clones (Supplemental Figure 1), BAC clones OO_Ba0120J21 from *O. officinalis* and OM_Ba0293H21 from *O. minuta*, which had the greatest degree of overlap with other positive clones from the same genome, were sequenced.

Analysis of approximately 113-kb sequences of clone OO_Ba0120J21 identified four putative genes that belonged to the *Xa3/Xa26* gene family. Three of the four paralogs in *O. officinalis* were orthologs of the *Xa3/Xa26* gene family *TRKa*, *TRKb*, and *TRKf* in Asian rice cultivar Teqing (*O. sativa* L. ssp. *indica*; Sun et al., 2006), respectively, according to their sequence similarity, corresponding locations within the family, and transcriptional orientation; they were thus named *OoRKa*, *OoRKb1*, and *OoRKf* (Figure 1). The fourth paralog in *O. officinalis* showed very high sequence similarity with *OoRKb1* (94%) and *TRKb* (94%); it could be generated through tandem duplication or unequal crossover and thus was named *OoRKb2*.

Analysis of approximately 149 kb of sequence of clone OM_Ba0293H21 identified five putative genes that showed sequence homology with the *Xa3/Xa26* family orthologs. The five paralogs spanned an approximately 50-kb region (Figure 1) and four of them showed a high degree of sequence similarity respective to the *Xa3/Xa26* family orthologs *TRKa*, *TRKb*, *TRKg*, and *TRKc* in rice cultivar Teqing; they were designated *OmRKa*, *OmRKb1*, *OmRKg*, and *OmRKc*. The fifth paralog in *O. minuta* showed very high sequence similarity with *OoRKb2* (99%) and *OmRKb1* (94%); it was named *OmRKb2*. These results suggest that the origin of member b2 must have occurred before polyploidization.

OoRKb1, *OmRKa*, and *OmRKb1* were predicted to be intact genes, using *Xa3/Xa26* as the reference (Supplemental Figure 2). *OoRKa* and *OmRKc* were pseudogenes, with each containing a frame-shift mutation. *OoRKb2*, *OoRKf*, *OmRKb2*, and *OmRKg* were truncated as compared to *Xa3/Xa26* (Supplemental Figure 2). *OoRKb2* and *OmRKb2* appeared to encode only an incomplete kinase domain. *OoRKf* lost the predicted intron and the following sequence of this family and putatively encoded an intact LRR domain, but an incomplete kinase domain. *OmRKg* contained an in-frame stop codon and putatively encoded an incomplete LRR domain. The orthologs a, b, and c in rice cultivar Teqing are intact genes; the orthologs f and g in Teqing are pseudogenes with insertion, deletion, or in-frame stop codon, which are different from the mutations in *OoRKf* and *OmRKg* (Sun et al., 2006) (Supplemental Figure 2).

Comparing the sequences of *Xa3/Xa26* family orthologs in the two wild *Oryza* species to four Asian rice cultivars, Minghui 63 (*O. sativa* L. ssp. *indica*), Teqing, 93–11 (*O. sativa* L. ssp. *indica*), and Nipponbare (*O. sativa* L. ssp. *japonica*) (Sun et al., 2006), it was easy to recognize that they had very low sequence similarity (<49% sequence identity) between cultivated rice and wild *Oryza* species in the intergenic regions. However, nearly 34-kb regions with 95% sequence identity in the *Xa3/*

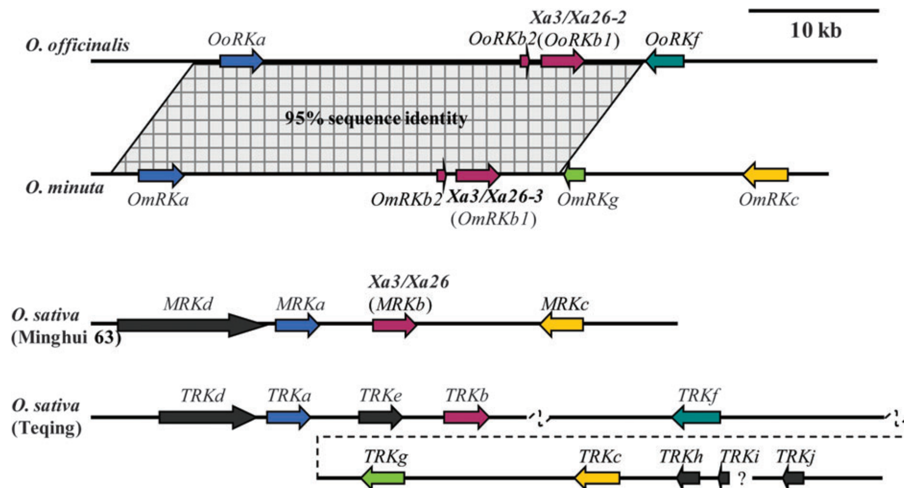

Figure 1. Comparison of Organization of the *Xa3/Xa26* Gene Family in Two Wild *Oryza* Species, *O. officinalis* and *O. minuta*, with that in Cultivated Rice Varieties, Minghui 63 and Teqing.
Members of the *Xa3/Xa26* family and their transcription orientation are indicated by arrows. Regions showing extreme sequence identity (95% DNA identity overall) between the two wild species are shadowed. Organization of the *Xa3/Xa26* family in Minghui 63 and Teqing is drawn according to published data (Sun et al., 2004, 2006); the dotted slant indicates the border between two sequence contigs; the question mark indicates that the neighborhood of two adjacent contigs is deduced according to sequence similarity and the *Xa3/Xa26* family organization in other cultivated rice varieties.

Xa26 family were identified between *O. officinalis* and *O. minuta*, which harbored orthologs a, b1, and b2 (Figure 1). These results suggest that the *Xa3/Xa26* families in the two wild *Oryza* species are less diverse between each other than as compared to the diversity found between them and cultivars.

Xa3/Xa26 Orthologs of the Wide *Oryza* Species Were Functional Disease Resistance Genes

OoRKb1 and *OmRKb1* were the orthologs of the *R* gene *Xa3/Xa26*. To determine whether *OoRKb1* and *OmRKb1* were functional in the rice–*Xoo* interaction, genomic fragments containing *OoRKb1* and *OmRKb1* with their native promoters were individually transformed into rice cultivar Mudanjiang 8, which is susceptible to *Xoo* (Supplemental Figure 3). Thirty-one independent positive transformants carrying *OoRKb1* (named D101OM) and 18 independent positive transformants carrying *OmRKb1* (named D103OM) were obtained. Eleven of the T_0 plants transformed with *OoRKb1* construct showed significantly ($P < 0.01$) enhanced resistance to *Xoo* strain PXO61, with lesion areas ranging from 3.4 to 35.0%, compared to 50.3% for wild-type Mudanjiang 8; another 10 of the T_0 plants transformed with the same construct showed significantly ($P < 0.01$) enhanced resistance to *Xoo* strain PXO341, with lesion areas ranging from 1.6 to 44.4%, compared to 61.2% for wild-type Mudanjiang 8 (Supplemental Table 1). All 18 T_0 plants transformed with *OmRKb1* construct showed significantly ($P < 0.01$) enhanced resistance to *Xoo* strain PXO61, with lesion areas ranging from 6.7 to 27.5%, compared to 55.2% for wild-type Mudanjiang 8 (Supplemental Table 1).

Two T_1 families from resistant T_0 (D101OM1 and D101OM43) and Two T_1 families from resistant T_0 (D103OM25 and D103OM43) were further analyzed for resistance to PXO61, and for the existence of the transgenic marker gene *GUS* that was tightly linked to the targeted transgene. The results showed that the enhanced resistance was associated with the presence of *OoRKb1/GUS* or *OmRKb1/GUS* in the T_1 families (Figure 2). The bacterial growth rates in *OoRKb1*- and *OmRKb1*-carrying plants were 7–51-fold and 13–153-fold lower than that in wild-type at the booting (panicle development) stage and 4–8-fold and 2–5-fold lower than that in wild-type at six-leaf stage at 6–12 d after infection, respectively (Figure 3). These results suggest that *OoRKb1* and *OmRKb1* confer *Xoo* resistance. Thus, we designated them *Xa3/Xa26-2* (*OoRKb1*; GenBank accession number: HQ148674) and *Xa3/Xa26-3* (*OmRKb1*; GenBank accession number: HQ148675) based on the naming system of rice *R* genes against *Xoo*.

Xa3/Xa26-2 and *Xa3/Xa26-3* Mediated Broad-Spectrum Resistance

Transgenic plants carrying a single copy of *Xa3/Xa26-2* or *Xa3/Xa26-3* were examined for their responses to different *Xoo* strains. The resistance of the transgenic plants to 16 of the 18 strains was significantly enhanced as compared to the wild-type Mudanjiang 8 at adult stage (Figure 4). The lesion areas of the transgenic plants carrying *Xa3/Xa26-2* and plants carrying *Xa3/Xa26-3* to the 16 *Xoo* strains were 31–92% and 15–91% smaller than those of the wild-type plants, respectively. The plants carrying *Xa3/Xa26-2*, and the plants carrying *Xa3/Xa26-3*, showed a similar resistance spectrum to the transgenic rice line Rb49, which carried a single copy of *Xa3/Xa26* driven by its native promoter with the same genetic background as the transgenic plants carrying *Xa3/Xa26-2* or *Xa3/*

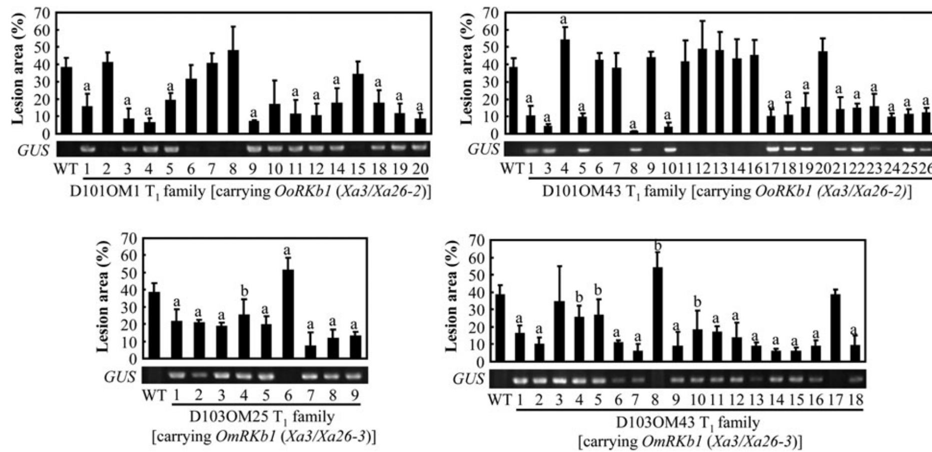

Figure 2. Enhanced Resistance to *Xoo* Strain PXO61 Associated with the Presence of a *GUS* Marker Gene that Was Tightly Linked to *OoRKb1* or *OmRKb1* in T_1 Families at Booting Stage.
Bars represent mean (three to five replicates) ± standard deviation. The 'a' or 'b' indicates that a significant difference between transgenic and wild-type (WT) Mudanjiang 8 was detected at $P < 0.01$ or $P < 0.05$, respectively.

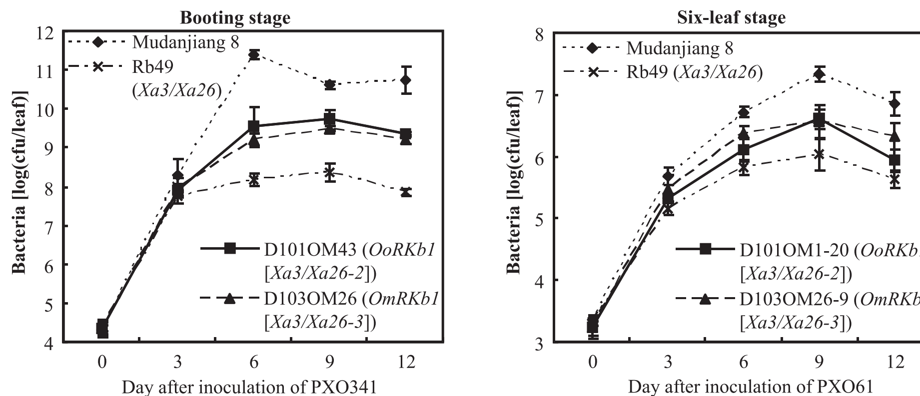

Figure 3. Growth of *Xoo* Strains PXO341 and PXO61 in Leaves of *OoRKb1*-Carrying (D101OM) and *OmRKb1*-Carrying (D103OM) Plants at Adult (Booting) Stage (T_1 Plants) and Seedling (Six-Leaf) Stage (T_3 Homozygous Lines).
Bacterial populations were determined from three leaves at each time point by counting colony-forming units (cfu). Mudanjiang 8 was wild-type. Rb49 was a transgenic line carrying *Xa3/Xa26* regulated by its native promoter in Mudanjiang 8 background.

Xa26-3 (Cao et al., 2007a). However, the resistance level of plants carrying *Xa3/Xa26* was significantly higher than plants carrying *Xa3/Xa26-2* or *Xa3/Xa26-3* to all 16 *Xoo* strains, which showed incompatible reactions with all the transgenic plants (Figure 4). Although the Rb49 line was compatible with *Xoo* strain PXO99, plants carrying *Xa3/Xa26-2* and plants carrying *Xa3/Xa26-3* appeared to be more susceptible to PXO99 than the Rb49. In addition, plants carrying *Xa3/Xa26-2* appeared to have a higher level of resistance to some of the *Xoo* strains than plants carrying *Xa3/Xa26-3* (Figure 4). *Xa3/Xa26-2*- and *Xa3/Xa26-3*-carrying plants were also showed enhanced resistance to different *Xoo* strains at seedling stage, but the resistance level was significantly lower than that of *Xa3/Xa26*-carrying plants (Figure 4). Consistent with these results, bacterial growth rates in *Xa3/Xa26-2*- and *Xa3/Xa26-3*-carrying plants were also higher than that in *Xa3/Xa26*-carrying plants (Figure 3). These results suggest that *Xa3/Xa26-2* and *Xa3/Xa26-3* can mediate a broad-spectrum resistance to *Xoo* as their ortholog *Xa3/Xa26*.

The two wild *Oryza* accessions used for constructing the BAC libraries were resistance to *Xoo* (Brar and Khush, 2002). We inoculated *O. officinalis* (accession 100896) and *O. minuta* (accession 101141), which were used for construction of the BAC libraries, with three Philippine *Xoo* races, PXO61, PXO99, and PXO341. They both present a high-level resistance to these *Xoo* strains as compared to susceptible rice variety Mudanjiang 8 (Table 1). Transgenic plants carrying *Xa3/Xa26-2* or *Xa3/Xa26-3* showed enhanced resistance to PXO61 and PXO341 but susceptibility to PXO99 as the transgenic line (Rb49) carrying *Xa3/Xa26* (Figure 4). These results suggest that the resistance of the two wild *Oryza* species to *Xoo* is at least partly contributed by *Xa3/Xa26-2* or *Xa3/Xa26-3*. However, they may also carry other *R* genes against *Xoo* in addition to *Xa3/Xa26-2* or *Xa3/Xa26-3*.

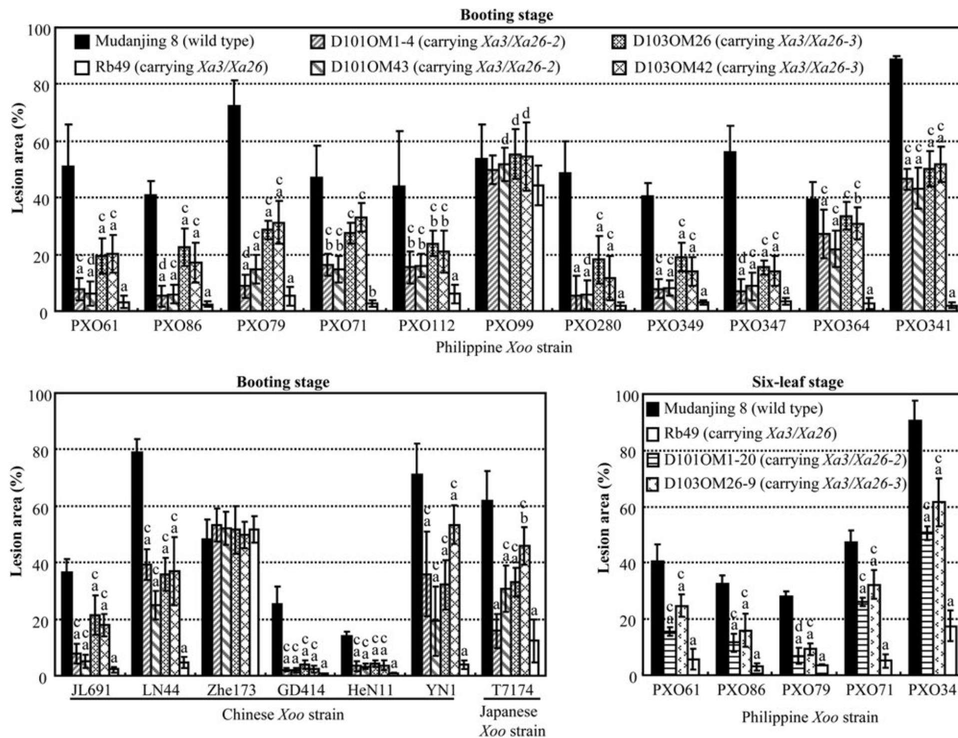

Figure 4. Resistance Spectrum of *Xa3/Xa26-2* and *Xa3/Xa26-3* to *Xoo* at Adult (Booting) and Seedling (Six-Leaf) Stages.
All the transgenic plants carried a single copy of transgene. D101OM43, D103OM26, and D103OM42 are resistance T_1 transgenic plants. D101OM1-4 is resistance T_2 transgenic plants. D101OM1-20 and M103OM26-9 are homozygous resistance transgenic lines (T_3 generation). Rb49 is a resistance transgenic line carrying *Xa3/Xa26* regulated by its native promoter with the genetic background of Mudanjiang 8 (Cao et al. 2007a). Bars represent mean (6–10 replicates for D101OM and D103OM and 3–5 replicates for Rb49 and wild-type at booting stage and 3–6 replicates at six-leaf stage) ± standard deviation. The 'a' or 'b' indicates that a significant difference between transgenic and wild-type was detected at $P < 0.01$ or $P < 0.05$, respectively. The 'c' or 'd' indicates that a significant difference between the *Xa3/Xa26-2*-carrying or *Xa3/Xa26-3*-carrying plants and *Xa3/Xa26*-carrying plants was detected at $P < 0.01$ or $P < 0.05$, respectively.

Table 1. Resistance of Two Wild *Oryza* Species to *Xoo* as Compared to Cultivated Rice Mudanjiang 8.[1]

Xoo strain	Mudanjiang 8	*O. officinalis* (accession 100896)[2]	*O. minuta* (accession 101141)[2]	Mudanjiang 8	Rb49 (*Xa3/Xa26*, Mudanjiang 8 background)[3]
PXO61	55.3 ± 16.0	2.7 ± 0.9[a]	1.4 ± 0.5[a]	50.9 ± 14.7	3.1 ± 2.1[a]
PXO99	57.7 ± 14.8	8.6 ± 2.1[a]	2.4 ± 0.2[a]	53.8 ± 11.8	44.5 ± 7.0
PXO341	81.6 ± 6.0	9.9 ± 3.4[a]	1.5 ± 1.0[a]	88.7 ± 1.0	2.1 ± 0.8[a]

1 Three to five uppermost fully expanded leaves of each plant were inoculated. Data represent mean (three to five replicates) ± standard deviation. The 'a' indicates that a significant difference between wild species or rice transgenic line Rb49 and susceptible rice Mudanjiang 8 (control) was detected at $P < 0.01$.
2 The lesion areas (%) were measured 23 d after inoculation.
3 The lesion areas (%) were measured 14 d after inoculation of PXO61 and PXO99 and 12 d after inoculation of PXO341.

Variations of Amino-Acid Sequences Encoded by *Xa3/Xa26* Orthologs

Aligning the genomic and cDNA sequences of *Xa3/Xa26-2* and *Xa3/Xa26-3* showed that the two orthologs had similar structures to *Xa3/Xa26*: two exons and one intron that was inserted in the region encoding the kinase domain (Figure 5). *Xa3/Xa26-2* and *Xa3/Xa26-3* had nearly the same structure: the same sizes of exons and 5′- and 3′-untranslated regions. The only difference between the two orthologs was that they had different sizes of introns (103 versus 104 nucleotides). Comparative sequence analyses of the coding regions and introns of *Xa3/Xa26*, *Xa3/Xa26-2*, and *Xa3/Xa26-3* revealed 82–99% identity among the three orthologs.

Both *Xa3/Xa26-2* and *Xa3/Xa26-3* encode proteins that consisted of 1092 amino acids as compared to 1 103 amino acids encoded by *Xa3/Xa26*. The predicted proteins encoded by

the three orthologs share 91–99% sequence identity and 94–99% sequence similarity. Xa3/Xa26-2 and Xa3/Xa26-3 proteins share 98% sequence identity and have only a total of 14 amino-acid residues distinct from each other (Figure 6). Of these 14 amino-acid changes, four are located in the region in front of the LRR domain, seven in the LRR domain, and three in the kinase domain.

Figure 5. Comparison of the Structures of *Xa3/Xa26-2* and *Xa3/Xa26-3* with *Xa3/Xa26*.
The coding regions (black boxes) of the genes are interrupted by one intron (line). The positions of 5′ and 3′ UTR (white boxes), translation start codon (ATG), and translation stop codon (TGA) are also indicated. The numbers indicate the nucleotides of each substructure.

Both Xa3/Xa26-2 and Xa3/Xa26-3 proteins share 91% sequence identity to Xa3/Xa26 protein. The two proteins have 88 and 90 amino-acid difference from Xa3/Xa26, respectively (Figure 6). Of these 88 and 90 amino-acid changes, 11 are amino-acid deletions (eight in the regions in front of the LRR domain, two in the kinase domains, and one in the juxtamembrane regions) in the same sites of Xa3/Xa26-2 and Xa3/Xa26-3. Of the 77 and 79 total amino-acid substitutions compared to Xa3/Xa26, approximately half of them (39 and 38) are in the LRR domains of Xa3/Xa26-2 and Xa3/Xa26-3, respectively. Most of the substitution sites are the same in the LRR domains of the two proteins. Compared to Xa3/Xa26, the distributions of the amino-acid changes in the LRR regions of Xa3/Xa26-2 and Xa3/Xa26-3 are dispersed. Nearly one-third of the variants in the LRR regions lie within the xxLxLxx ('x' indicates any amino-acid residue) motifs. Xa3/Xa26-2 and Xa3/Xa26-3, compared to Xa3/Xa26, have the same nine substitutions in the transmembrane and juxtamembrane regions. Nearly half of the remaining variants are concentrated in the regions in front of the LRR domain, within an approximate a range of 80 amino acids. The rest of the variants are dispersed in the kinase domains of Xa3/Xa26-2 and Xa3/Xa26-3 proteins. These results suggest that *Xa3/Xa26-2* and *Xa3/Xa26-3* are more evolutionarily related to each other than their evolutionary relationship with *Xa3/Xa26*.

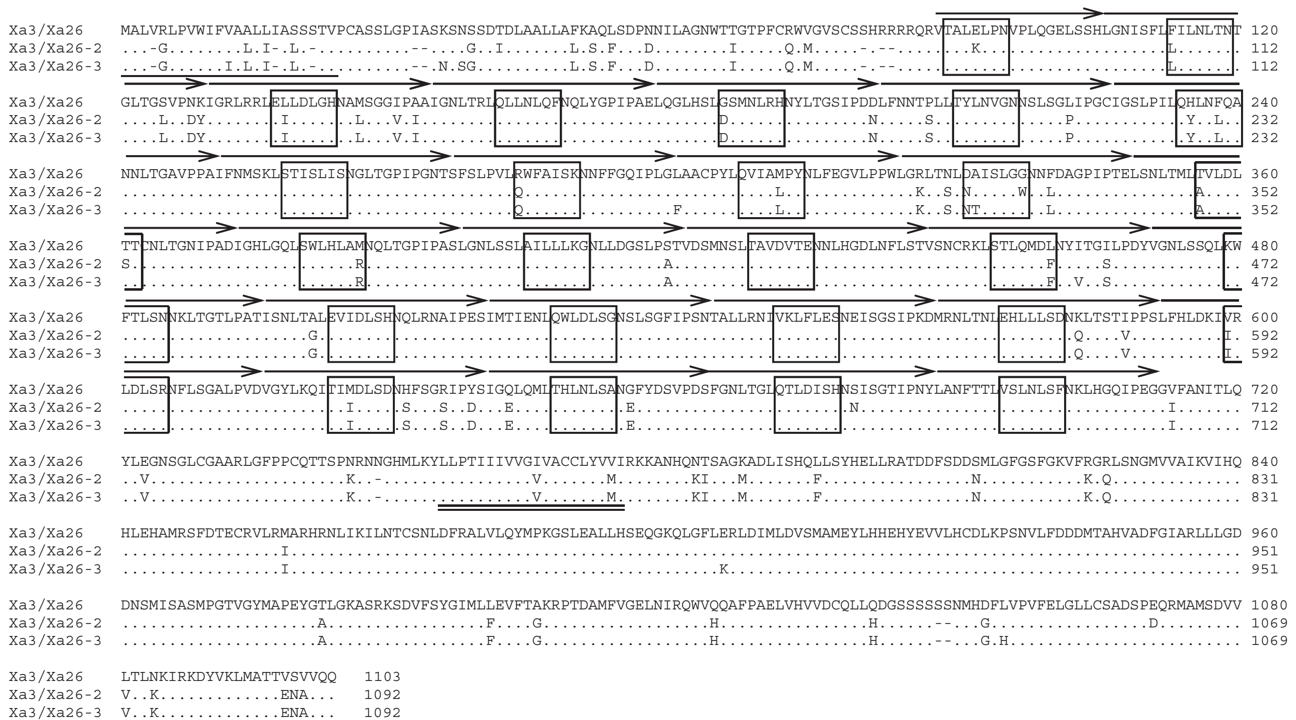

Figure 6. Alignment of Xa3/Xa26-2, Xa3/Xa26-3, and Xa3/Xa26 Proteins.
All sequences are compared with the reference Xa3/Xa26. The predicted signal peptide sequence is underlined. The predicted transmembrane region is double underlined. The arrows above the amino-acid residues indicate the LRR repeats and the xxLxLxx motifs of the LRR domain are boxed. Dots represent identical amino acids of Xa3/Xa26-2 and Xa3/Xa26-3 to Xa3/Xa26. The dash represents the single amino acid absent in Xa3/Xa26-2 and Xa3/Xa26-3.

DISCUSSION

The *Xa3/Xa26* family is a potential disease resistance gene reservoir. In addition to *Xa3/Xa26*, the *MRKa*, a paralog of *Xa3/Xa26* in rice cultivar Minghui 63, can mediate *Xoo* resistance when enhancing its expression (Cao et al., 2007b). Other paralogs of this family in different Asian rice cultivars are expressed in rice leaves, which are one of the major invasion sites of pathogens, suggesting their potential role in rice–pathogen interactions (Xu et al., 2007). The present results further support the assumption that the *Xa3/Xa26* family is rich in genes for disease resistance.

It is generally accepted that the LRR domains of R proteins function directly or indirectly in recognition of pathogen effectors and play an important role in race-specific resistance (Rivas and Thomas, 2005; Ellis et al., 2007). Comparative sequence analysis of *Xa3/Xa26* family paralogs in four rice cultivars reveals that positive selection of point mutations in the LRR domains is a major force for the evolution of this family, suggesting the important roles of LRR domains in the functions of these family members (Sun et al., 2006). Domain swapping analysis further supports this hypothesis; this analysis has revealed that the LRR domain of Xa3/Xa26 protein is a major determinant of race-specific recognition during rice–*Xoo* interaction (Zhao et al., 2009). Furthermore, the juxtamembrane region of Xa3/Xa26 protein also appears to contribute to resistance specificity (Zhao et al., 2009). In addition, genetic background also influences the resistance spectrum and resistance level conferred by Xa3/Xa26 (Cao et al., 2007a; Zhou et al., 2009). Analysis of the predicted amino-acid sequences of Xa3/Xa26-2 and Xa3/Xa26-3 revealed the same known motif (transmembrane region) and domains (LRR and kinase) with Xa3/Xa26 (Figure 6). Xa3/Xa26-2 and Xa3/Xa26-3 also harbor 26 imperfect LRRs with consensus sequence of L/IxxLxxLxxLxLxxNxLxGxIPxx for LRR ('x' indicating any amino acid) as Xa3/Xa26. Although more than 40% of the amino-acid diversity residues between Xa3/Xa26-2 or Xa3/Xa26-3 and Xa3/Xa26 occur in the LRR domain, even in the xxLxLxx motif that forms the solvent-exposed surface for pathogen recognition in the LRR domain (Padmanabhan et al., 2009), plants carrying *Xa3/Xa26-2* or *Xa3/Xa26-3* showed a similar resistance spectrum to plants carrying *Xa3/Xa26*. These results suggest that these polymorphic amino-acid residues in Xa3/Xa26-2 and Xa3/Xa26-3 proteins, compared to Xa3/Xa26 protein, are not at the core region for pathogen recognition specificity. However, further study is required to determine whether some of these amino-acid changes may influence the level of resistance.

O. officinalis and *O. minuta* belong to the *O. officinalis* complex, and both species carry the C genome. *O. minuta* is a BBCC genome allotetraploid and is thought to have arisen from a hybridization between *O. officinalis*, a CC genome diploid, and an extinct BB diploid species (Wang et al., 2009). Comparative sequence analysis revealed a high degree of sequence homology of *Xa3/Xa26* families between diploid *O. officinalis* and tetraploid *O. minuta*, suggesting that the analyzed BAC clone containing *Xa3/Xa26* family in *O. minuta* might have originated from the CC genome. Thus, both *Xa3/Xa26-2* and *Xa3/Xa26-3* belong to the CC genome. *Xa3/Xa26* was first isolated from rice cultivar Minghui 63 (AA genome) (Sun et al., 2004). Minghui 63 has the pedigree of *O. nivara*, an AA genome wild species (Xie, 1998; Khush and Virk, 2005). Comparison of the *Xa3/Xa26* family sequences in Minghui 63 (Sun et al., 2006) and *O. nivara* (Li and Wang, unpublished data) revealed that *Xa3/Xa26* and its paralog *MRKc* as well as the intergenic region between *Xa3/Xa26* and *MRKc* in Minghui 63 share 98, 98, and 97% sequence identify to their orthologs and the corresponding intergenic region in *O. nivara*, respectively. However, the ortholog of *Xa3/Xa26* in the *O. nivara* accession used for sequencing is a pseudogene with an in-frame stop codon. This comparison suggests that the region harboring *Xa3/Xa26* in Minghui 63 might have been introduced from an *O. nivara* accession that was similar to the accession used for sequencing. The AA and CC genome lineages diversified ~7.5 million years ago (Ammiraju et al., 2008; Lu et al., 2009; Ammiraju et al., 2010; Sanyal et al., 2010). The orthologs at *Xa3/Xa26* locus in different *Oryza* species confers a similar resistance spectrum, which may imply that this resistance locus appeared earlier than the divergence of the AA and CC genomes. Thus, the resistance function of *Xa3/Xa26* locus appears to be relatively conserved during evolution.

Xa3/Xa26 family proteins belong to the same type of LRR-receptor kinase proteins as rice Xa21 protein, which also mediates resistance to *Xoo* (Sun et al., 2004). Xa21 functions both as an R protein and as a pattern recognition receptor (PRR) by recognition of an evolutionarily conserved pathogen-associated molecular pattern, a sulfated peptide (Lee et al., 2009). Another well-studied PRR is *Arabidopsis* FLS2, which also encodes a LRR-receptor kinase (Gómez-Gómez and Boller, 2000; Ali and Reddy, 2008). The *Xa3/Xa26*, *Xa3/Xa26-2*, and *Xa3/Xa26-3* from the two diverged genomes mediate similar resistance spectrums. It remains to be elucidated whether the encoding proteins of *Xa3/Xa26* and its alleles perceive the same conserved pathogen component in rice–*Xoo* interaction.

Durable resistance refers to resistance that remains effective during its prolonged and widespread use in environments favorable to pathogen or disease spread (Johnson, 1981). The *indica* rice cultivar Minghui 63, carrying *Xa3/Xa26*, is a parent of a set of hybrids that account for more than 20% of total rice production area in China for the last two decades. In addition, *Xa3/Xa26* is also an important resistance gene in *japonica* cultivar breeding in China (Xu et al., 2004). The two wild *Oryza* accessions used both presented a high level of resistance to *Xoo* strains PXO61, PXO99, and PXO341. However, transgenic plants carrying *Xa3/Xa26-2* or *Xa3/Xa26-3* showed enhanced resistance to PXO61 and PXO341 but susceptibility to PXO99 as the transgenic line (Rb49) carrying *Xa3/Xa26*. These results indicated that there is another *R* gene(s) in the two wild *Oryza* species against PXO99. Although the CC and BBCC wild rice species have not been cultivated in large areas, the orthologs, originated at least 7.5 million years ago, still remain functionally in the same genomes as other *R* genes together by the long

natural selection. The wide use of *Xa3/Xa26*-carrying cultivars in rice production and the similar resistance specificity of the ancient *Xa3/Xa26-2* and *Xa3/Xa26-3* as the present *Xa3/Xa26* suggest that this *R* gene locus may confer durable resistance in addition to conferring a relative broad-spectrum resistance.

METHODS

Selection of BAC Clone

Mixed probes of DNA segments of *Xa3/Xa26* and its paralog *MRKa* from rice cultivar Minghui 63 (*Oryza sativa* ssp. *indica*; Cao et al., 2007b) were used to screen the *O. officinalis* and *O. minuta* BAC libraries (Ammiraju et al., 2006). DNA segments corresponding to the LRR domain of *MRKa* and the kinase domain of *Xa3/Xa26* were amplified using gene-specific primers (Supplemental Table 2). Positive BACs were digested with restriction enzyme *Hin*dIII, transferred to nylon membranes, and hybridized separately using *Xa3/Xa26* and *MRKa* DNA probes. BAC that has the most hybridizing bands in each genome was sequenced.

BAC Sequencing and Sequence Assembly

BAC clones were sequenced using a shotgun strategy. To construct a subclone library for sequencing, DNA from each BAC clone was randomly sheared by sonication. DNA fragments in the 2–4-kb size frame were size-selected, blunt-ended, and then ligated into the pUC19 vector. Clones were sequenced from both directions using M13 universal forward and reverse primers and BigDye Terminator v3.0 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA). Sequence reads were assembled with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA). Gaps were filled by a combination of primer walking and shotgun sequencing of subclones with extremes at both sides of the sequencing gaps.

Sequence Annotation and Computational Analysis

Assembled BAC sequences were annotated by using the gene prediction program Fgenesh (http://morissardjerome.free.fr/infobiogen/www.softberry.com/berry.html) (Salamov and Solovyev, 2000) and BLAST (Blastn, Blastx and Blastp) analyses against different databases (Altschul et al., 1997). Pair-wise sequence comparisons were carried out using YASS program (http://bioinfo.lifl.fr; Noe and Kucherov, 2005) and the Global Sequence Alignment Tool (www.ncbi.nlm.nih.gov/blast/Blast.cgi; Needleman and Wunsch, 1970). ClustalX (Thompson et al., 1997) was used for multiple sequence alignment.

Gene Cloning and Rice Transformation

BAC clones OO_Ba0120J21 from *O. officinalis* and OM_Ba0293H21 from *O. minuta* were digested with restriction enzymes *Eco*RV and *Sam*I, respectively. An 11.9-kb DNA fragment harboring *OoRKb1* and its native promoter from OO_Ba0120J21 and a 13.7-kb DNA fragment harboring *OmRKb1* and its native promoter from OM_Ba0293H21 were individually ligated with the vector pCAMBIA1301 digested with restriction enzyme *Sma*I (Supplemental Figure 2). The constructs containing *OoRKb1* and *OmRKb1* were transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium*-mediated transformation was performed using calli derived from mature embryos of susceptible rice cultivars Mudanjiang 8 (*O. sativa* L. ssp. *japonica*) according to a published procedure (Lin and Zhang, 2005). Positive transgenic plants were identified by PCR amplification of the marker gene β-glucuronidase (*GUS*) using gene-specific primers (Supplemental Table 2).

Pathogen Inoculation and Disease Scoring

Three to five uppermost fully expanded leaves of each plant were inoculated with different *Xoo* strains using the leaf-clipping method (Chen et al., 2002) at six-leaf and booting stages. *Xoo* strains included Chinese strains GD414, HeN11, JL691, LN44, YN1, and ZHE173, Japanese strain T7174, and Philippine strains PXO61 (race 1), PXO86 (race 2), PXO79 (race 3), PXO71 (race 4), PXO112 (race 5), PXO99 (race 6), PXO280 (race 8), PXO349 (race 9b), PXO347 (race 9c), PXO364 (race 9d), and PXO341 (race 10). Because cultivated rice and wild *Oryza* species had different leaf length, disease was scored by measuring percent lesion area (lesion length/leaf length) 2–3 weeks after inoculation. The bacterial growth rate in rice leaves was determined by counting colony-forming units (Sun et al., 2004).

Gene Structure Analysis

Total RNA extracted from the leaves of resistant T_1 transgenic plants was used to analyze the structures of the transgenes. The 5' and 3' end cDNA sequences of target genes were determined by rapid amplification of cDNA ends (RACE) using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions (Qiu et al., 2007). Intermediate cDNA fragments of the transgenes were obtained by reverse transcription (RT)–PCR. The primers used for RACE and RT–PCR analyses are listed in Supplemental Table 1. The RACE and RT–PCR products were ligated into the pGEM–T Easy vector (Promega, Madison, WI, USA) and sequenced.

Statistical Analyses

The significant differences between the samples of transgenic and wild-type plants were analyzed by the pair-wise *t*-test installed in the Microsoft Office Excel program.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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