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Xa26, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein

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Summary

Rice bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most serious rice diseases worldwide. A rice gene, *Xa26*, conferring resistance against *Xoo* at both seedling and adult stages was isolated by map-based cloning strategies from the rice cultivar Minghui 63. *Xa26* belongs to a multigene family consisting of four members. It encodes a leucine-rich repeat (LRR) receptor kinase-like protein and is constitutively expressed. Sequence analysis revealed that IRBB3 and Zhachanglong lines that are resistant to a broad range of *Xoo* strains, also carry *Xa26*. However, significant difference in lesion length was observed among these lines after inoculation with a set of *Xoo* strains. Moreover, transgenic plants carrying *Xa26* showed enhanced resistance compared with the donor line of the gene in both seedling and adult stages. These results suggest that the resistance conferred by *Xa26* is influenced by the genetic background.

Keywords: *R* gene, *Xoo*, bacterial blight disease, receptor kinase protein, multigene family, rice.

Introduction

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases of rice worldwide. Utilization of resistance (*R*) genes in breeding programs has been the most effective and economical strategy for controlling bacterial blight. Currently, more than 20 genes conferring host resistance against various strains of *Xoo* have been identified (Chen *et al.*, 2002; Khush and Angeles, 1999; Lee *et al.*, 2003; Lin *et al.*, 1996; Nagato and Yoshimura, 1998; Yang *et al.*, 2003; Zhang *et al.*, 1998). However, only two of the resistance genes, *Xa21* and *Xa1*, have been isolated. It has been shown that *Xa21* encodes a leucine-rich repeat (LRR) receptor kinase-like protein (Song *et al.*, 1995), while *Xa1* encodes a nucleotide-binding site (NBS)-LRR protein (Yoshimura *et al.*, 1998).

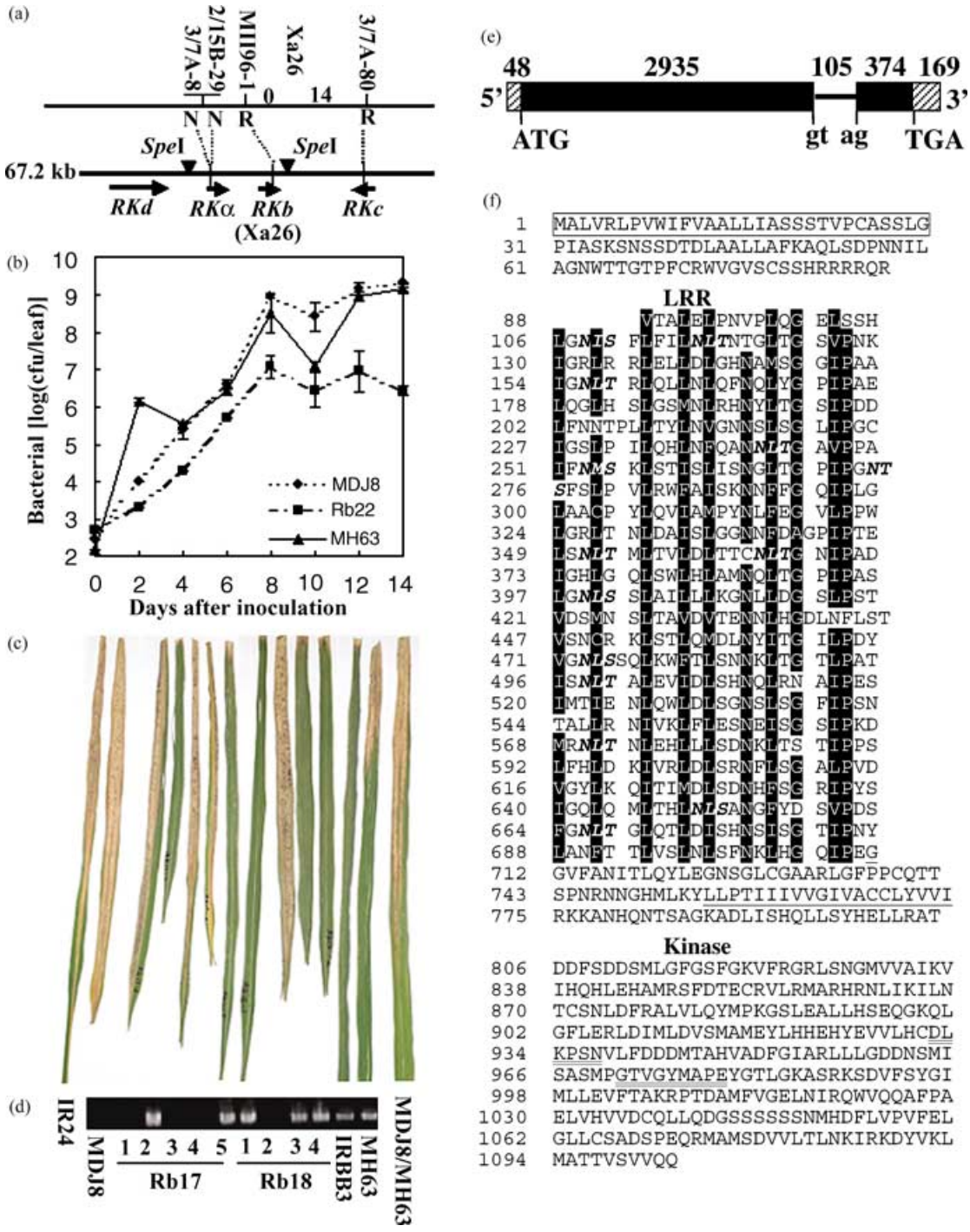
Xa21 provides resistance to a broad spectrum of *Xoo* strains (Chen *et al.*, 2000; Khush *et al.*, 1990; Wang *et al.*, 1996). However, like several other bacterial blight resistance genes (Century *et al.*, 1999; Goel and Gupta, 1990; Mew, 1987; Ogawa, 1993; Zhang and Mew, 1985), the resistance activity of *Xa21* is developmentally controlled; its resistance increases progressively from being susceptible at the juvenile stage to fully resistant at the adult stage.

The function of *R* gene products as receptors interacting with pathogen elicitors in defense signaling has been suggested by direct and indirect evidences (Baker *et al.*, 1997; Jia *et al.*, 2000; Jones, 2001; Nimchuk *et al.*, 2001; Tang *et al.*, 1996). Limited studies have also shown that additional host factors are required for pathogen recognition in some *R* gene-mediated disease resistances. For example, the *Prf* gene product is required for the function of *Pto* gene against *Pseudomonas syringae* pathogen expressing avrPto in tomato (Martin *et al.*, 1993; Salmeron *et al.*, 1994). A recent study reveals that the function of an allele of *Arabidopsis R* gene *RPS2* is influenced by genetic background and the LRR domain determines the effectiveness of the interaction between *RPS2* and other host factors in *RPS2*-mediated resistance (Banerjee *et al.*, 2001). In rice, effect of genetic background on *R* gene-mediated resistance has also been indicated (Ogawa *et al.*, 1986a,b,c, 1988a).

We previously identified a dominant rice gene, *Xa26*, for *Xoo* resistance that was mapped to the long arm of

chromosome 11 within a genomic region of 20 kbp in length (Yang *et al.*, 2003). The rice germplasm carrying this gene is Minghui 63 (*Oryza sativa* ssp. *indica*), the restorer line of Shanyou 63, the most widely cultivated hybrid in China for more than two decades. Therefore, this gene has contributed greatly to the success of hybrid rice production. A characteristic of *Xa26* is its resistance activity against a number of *Xoo* strains at both seedling and adult stages, suggesting that this gene may not be developmentally regulated (Chen *et al.*, 2002).

We report here the isolation and characterization of *Xa26* and its family members. We showed that *Xa26* encodes a protein similar to the *Xa21* gene product. The function of the cloned *Xa26* was confirmed by transformation analysis.



Results

Sequence analysis of the Xa26 *region identified a multigene family*

The *Xa26* gene for *Xoo* resistance was previously localized to the bacterial artificial chromosome (BAC) clone 3H8 (about 95 kbp in size) identified from a Minghui 63 BAC library that carries *Xa26* (Yang *et al.*, 2003). It was also determined that the *Xa26* locus was flanked by two DNA fragments, 2/15B-29 and 3/7A-80, both subclones of 3H8. Sequence analysis of 3H8 generated two large sequence contigs. The larger contig, 67.2 kbp in length, contained the sequences of 2/15B-29 and 3/7A-80 (Figure 1a).

More than 10 genes were predicted in this 67.2-kbp fragment. BLASTX analysis showed that only four predicted genes had sequence homology with known plant *R* genes. They all encode LRR receptor kinase-like (RK) proteins and share various degrees of sequence similarities (Table 1). These four predicted genes were designated *RKa*, *RKb*, *RKc*, and *RKd* with their relative locations shown in Figure 1(a). *RKa*, *RKb*, and *RKc* were predicted to be intact genes and had similar structures consisting of two exons and one short intron (Figure S1). The three genes had a higher sequence similarity in the predicted coding region than in the predicted intron (Table 1). *RKd* was also predicted as consisting of two exons and one intron, but it was interrupted by two large fragments. One fragment, 5881 bp in size with partial homology to rice Epstein-Barr virus nuclear antigen (EBNA)-1-like protein (GenBank Accession number: BAC56827; *E*-value $= 3e^{-39}$), was inserted in the predicted coding region; another fragment, 2188 bp in size with no significant homology with any sequences in GenBank, was inserted in the predicted intron region (Figure S1). In addition, two frameshift sites and one nonsense mutant site also occurred in the coding region (Figure S1). These results suggest that *RKd* has a truncated open-reading frame or is a pseudogene. Based on their sequence similarity and genomic locations, *RKa*, *RKb*, *RKc*, and *RKd* were regarded as belonging to a multigene family.

Table 1 Analysis of the sequence identify (%) among member of the *Xa26* gene family[a]

	RKb	*RKc*	*RKd*
RKa	78/35	62/33	76/46
RKb		62/27	74/60
RKc			60/27

[a]Predicted coding region/intron.

Sequence analysis showed that two subclones of BAC 3H8, 3/7A-8 and 2/15B-29, contained part of the sequence of *RKa*, another subclone, M196-1, had part of the sequence of *RKb*, and still another subclone, 3/7A-80, was a part of *RKc* (Figure 1a). As described in our previous study, 3/7A-8 and 2/15B-29 detected non-parental banding patterns in five resistant lines from a recombinant inbred population segregating for the *Xa26* gene (Yang *et al.*, 2003). This indicates that alteration of *RKa* did not abolish the function of *Xa26*, and therefore, *RKa* is a poor candidate for *Xa26*. Probes prepared of M196-1 and 3/7A-80, respectively, detected the banding patterns of the resistant parent (Minghui 63) in the same five lines. Moreover, M196-1 co-segregated with the *Xa26* locus, whereas recombination occurred between *Xa26* and 3/7A-80 in 14 of the 477 susceptible individuals selected from a large F_2 population segregating for *Xa26* (Yang *et al.*, 2003). These results strongly suggest that *RKb* was the best candidate for the *Xa26* gene.

Analysis of transgenic plants determined that RKb *is* Xa26

To determine which member of the gene family was *Xa26*, the genomic fragments containing *RKa*, *RKb*, and *RKc* with their native promoters were individually transferred by *Agrobacterium*-mediated transformation into rice cultivar

Figure 1. The *Xa26* gene and its encoding product.
(a) The upper part is the genetic map of *Xa26* resolved using the subclones of BAC 3H8 as molecular markers as presented previously by Yang *et al.* (2003). The numbers between the molecular markers indicate the number of recombination events between the *Xa26* locus and the markers detected using 477 highly susceptible plants from a F_2 population segregating for *Xa26*. The capital letters underneath the marker names indicate the band patterns detected by the subclones in the five lines (see text): R, band of the resistant parent; and N, non-parental band. The lower part shows the locations of the members *RKa*, *RKb* (*Xa26*), *RKc*, and *RKd* of the Xa26 gene family. The arrows indicate the positions and transcription orientations of the members. The dashed lines indicate the positions of the subclones relative to the members of the gene family. Arrowhead, *Spe*I restriction enzyme sites flanking *RKa* and *RKb*.
(b) Growth of *Xoo* strain PXO61 in leaves of T_2 plants (Rb22-3) of transgenic lines Rb22, and control lines, Minghui 63 (MH63) and Mudanjiang 8 (MDJ8). The bacterial populations were determined from two to five leaves at each time point by counting colony-forming units (CFUs). Leaves were sterilized using 75% ethanol, ground separately, suspended in sterilized water, and plated on potato agar media.
(c) Leaves from the T_1 families of transgenic lines Rb17 and Rb18, IRBB3, MH63, MDJ8, and IR24, and the F_1 of a cross between MDJ8 and MH63 at 15 days after inoculation with *Xoo* strain PXO61.
(d) The genotype of the plants was examined by PCR using *RKb*-specific primers, RKb-4F and RKb-3R. A resistant plant produced a band and a susceptible plant did not have band.
(e) The coding region (black boxes) of *Xa26* is interrupted by one intron (line). The positions of 5′ and 3′ UTR (hatched boxes), translation start codon (ATG), translation stop codon (TGA), 5′ and 3′ splice junctions (gt and ag) are also indicated. The numbers indicate the base pairs of each substructure.
(f) Predicted amino acid sequence of the *Xa26* gene product. The signal peptide sequence is boxed. The LRR domain consists of 26 imperfect repeats with 14 *N*-linked glycosylation site (NxS/T; italics in bold). The black-shaded residues in the LRR domain indicate the ones matching the consensus or are hydrophobic in the conserved hydrophobic sites. The predicted transmembrane region is underlined. In the kinase domain, the conserved regions are double underlined.

Mudanjiang 8 (*O. sativa* ssp. *japonica*) that was susceptible to *Xoo*. In total, 120, 119, and 20 independent transformants were generated with *RKa*, *RKb*, and *RKc*, respectively.

Minghui 63 (*Xa26*) was moderately resistant to Philippine *Xoo* strains PXO61 (race 1) and PXO86 (race 2) and susceptible to *Xoo* strain PXO71 at the booting stage (Yang *et al.*, 2003). The transgenic plants of the T_0 generation were inoculated with PXO61 or PXO86 at the booting stage.

Table 2 Co-segregation of resistance in T_1 families of six plants transformed with *RKb*[a]

T_1 plant/cultivar	Lesion length	*RKb*[d]	*Gus*[d]
Cultivar			
IR24	18.2 ± 3.5[a]		
	15.8 ± 0.4[b]		
Mudanjiang 8	16.2 ± 2.9[a]		
	8.0 ± 0.4[b]		
	19.2 ± 3.9[c]		
F_1[e]	11.4 ± 3.5[a]	+	
Minghui 63	12.3 ± 5.1[a]	+	
	23.9 ± 5.0[c]		
IRBB3	0.7 ± 0.6[a]	+	
	4.2 ± 1.5[b]		
Transgenic line Rb17 (2.9 ± 1.19)[b]			
1	16.9 ± 2.5[a]	−	−
2	0.2 ± 0.0[a]	+	+
3	16.2 ± 1.9[a]	−	−
4	12.5 ± 2.3[a]	−	−
5	0.6 ± 0.3[a]	+	+
Transgenic line Rb22 (0.9 ± 1.08)[b]			
1	16.7 ± 3.4[a]	−	−
2	0.6 ± 0.3[a]	+	+
3	0.2 ± 0.0[a]	+	+
4	1.3 ± 0.7[a]	+	+
5	1.0 ± 0.7[a]	+	+
6	0.7 ± 0.5[a]	+	+
7	18.1 ± 1.9[a]	−	−
8	2.0 ± 1.7[a]	+	+
9	1.2 ± 1.2[a]	+	+
10	1.4 ± 1.2[a]	+	+
Transgenic line Rb11 (0.3 ± 0.17)[b]			
1	0.5 ± 0.6[a]	+	
2	18.3 ± 2.5[a]	−	
3	14.0 ± 2.3[a]	−	
4	0.4 ± 0.3[a]	+	
5	1.4 ± 0.8[a]	+	
6	16.3 ± 5.6[a]	−	
7	16.7 ± 2.9[a]	−	
8	4.8 ± 2.6[a]	+	
9	0.6 ± 0.4[a]	+	
10	21.0 ± 1.4[a]	−	
11	16.2 ± 3.3[a]	−	
12	10.9 ± 3.4[a]	−	
13	1.0 ± 1.0[a]	+	
14	13.2 ± 3.1[a]	−	
15	0.6 ± 0.5[a]	+	
Transgenic line Rb16 (1.1 ± 0.87)[b]			
1	17.6 ± 2.4[a]	−	−
2	1.6 ± 1.4[a]	+	+
Transgenic line Rb18 (3.7 ± 0.74)[b]			
1	0.7 ± 0.6[a]	+	+
2	18.2 ± 2.9[a]	−	−
3	0.2 ± 0.0[a]	+	+
4	0.6 ± 0.6[a]	+	+
Transgenic line Rb49 (0.3 ± 0.20)[b]			
62 plants	0.2 − 3.2[c]	+	
18 plants	16.1 − 22.4[c]	−	

Four to seven uppermost fully expanded leaves of each plant were inoculated with *Xoo* strain at the booting stage. The lesion length (cm) was measured.
[a]Inoculation with *Xoo* strain PXO61.
[b]Inoculation with *Xoo* strain PXO86.
[c]Inoculation with *Xoo* strain PXO71.
[d]Determined by PCR amplification using *Gus*-specific primers and *RKb*-specific primers RKb-4F and RKb-3R for T_1 plants of line Rb49 and primers RKb-3′race1 and RKb-R for the rest T_1 plants and control cultivars.
[e]F_1 from the cross between Mudanjiang 8 and Minghui 63.

All the plants transformed with *RKa* (120) and *RKc* (20) were susceptible to PXO61, whereas 53 (67%) of the 79 tested plants transformed with *RKb* were highly resistant to PXO86. The average lesion length of the resistant transgenic plants was 0.7 ± 0.44 cm as compared to 8.0 ± 0.40 cm measured for the control of untransformed Mudanjiang 8. The bacterial growth analysis demonstrated that the growth rate of *Xoo* strain PXO61 on transgenic line Rb22 at the booting stage was 379- and 539-fold lower than that on Minghui 63 and Mudanjiang 8, respectively (Figure 1b).

To confirm that the resistance of the transgenic plants was because of *RKb* transgene, T_1 families derived from six of the resistant T_0 plants were examined individually for resistance by inoculating with *Xoo* strains PXO61 or PXO71, and also for the presence of the transgene by assaying *RKb* and the marker gene, β-glucuronidase (*Gus*), using PCR analysis. It was shown that the resistance co-segregated with *RKb* in all the six T_1 families (Table 2; Figure 1c,d), although the segregation in three of the families deviated from the expected 3 : 1 ratio.

Xa26 *encodes an LRR receptor kinase-like protein*

Analysis of the genomic sequence spanning *Xa26* with GENSCAN predicted that the *Xa26* gene (GenBank Accession number: AY364476) contains a coding region of 3309 bp interrupted by one intron of 105 bp (Figure 1e). Comparing the sequence of the reverse transcription-PCR (RT-PCR) product obtained using primers RKb-3′race2 and RKb-2R spanning the predicted intron of *RKb* with the

genomic sequence confirmed the position of the intron. Further analysis of the transcript of *Xa26* using 5′- and 3′-rapid amplification of cDNA end (RACE) assays also indicated that this gene contained one intron. The 5′ end of the transcript was 48 bp upstream of translation start codon, and the 3′ end of the transcript was 166 bp downstream of translation stop codon (Figure 1e).

Analysis of the predicted 1103 amino acid (aa) sequences of XA26 revealed several known regions and domains (Figure 1f). There is a signal peptide of 30 aa at the NH_2-terminus. The extracellular domain of XA26 consists of 26 imperfect LRRs at aa 88–711 with 14 *N*-linked glycosylation consensus sites (NxS/T; 'x' indicates any aa other than proline). The LRR consensus sequence of XA26 consists of 24 aa with a characteristic motif of L/IxxLxxLxxLxLxxNxLxGxIPxx ('x' indicates any aa). There is a transmembrane region located at aa 755–774. The aa 806–1103 region contains a putative cytoplasmic protein kinase domain. Two regions of the kinase domain are highly similar to the consensus sequences of subdomains VI (DLKPEN) and VIII (GT/SxxY/Fx APE) of serine/threonine protein kinase (Hanks *et al.*, 1988; Figure 1f).

Xa26 *is constitutively expressed*

The expression of the members of the Xa26 gene family was investigated by RT-PCR using total RNA from leaves of Minghui 63 at the booting stage inoculated with Chinese *Xoo* strain JL691 or Philippine *Xoo* strain PXO61. Member-specific primers were designed for each of *Xa26*, *RKa*, *RKc*, and *RKd* (Figure S1). For *Xa26* and *RKa*, the amplified fragments encompass the predicted splicing sites of the introns, which also served to distinguish amplified products of mRNA from contaminating genomic DNA in the RT-PCR. For *RKc*, the designed primers amplified only one of the two exons. Whereas, the primers designed for *RKd* amplified the putative LRR-encoding region. No detectable expression difference was observed for *Xa26* among different time points after inoculation with *Xoo* strains JL691 or PXO61 and between the *Xoo* and water inoculations (Figure 2a,b). Thus, it was concluded that the expression of *Xa26* is constitutive. Expression of *RKa* was also observed (Figure 2b), but no expression of *RKc* or *RKd* was detected (data not shown). Moreover, the expression pattern of *RKa* was not affected by inoculation of pathogen.

The expression of *Xa26* and *RKa* in different tissues and different growth stages of rice cultivar Minghui 63 was further examined by RT-PCR. The amplification product of one gene or the other was detected in leaf, sheath, and root tissues of different growth stages with varying levels of expression (Figure 2c), but neither was detected in the panicle tissue. *Xa26* was strongly expressed in leaves and weakly expressed in sheaths and roots with the highest expression detected in leaves of plants at the booting stage;

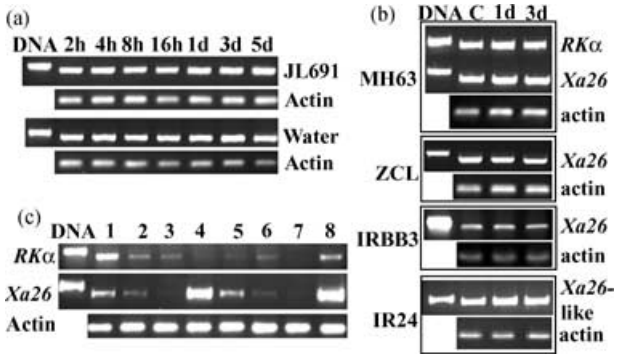


Figure 2. Expression patterns of *Xa26* and its family member *RKa* assayed by RT-PCR.
(a) The cultivar Minghui 63 (MH63) was inoculated with *Xoo* strain JL691 and water (control). Total RNA from 1 cm of leaf blades next to inoculation site was used for RT-PCR. The expression of the gene was examined at 2, 4, 8, and 16 h and 1, 3 and 5 days after inoculation.
(b) The expression of *Xa26* and *RKa* in resistant lines, MH63, Zhachanglong (ZCL), and IRBB3, and susceptible line IR24. The leaves of plants at the booting stage were inoculated with *Xoo* strain PXO61 or water (C). Total RNA from 4 to 5 cm of leaf blades next to inoculation site was used for RT-PCR.
(c) The expression of *Xa26* and *RKa* in different tissues from different growth stages of MH63. 1, leaf of seedling; 2, sheath of seedling; 3, root of seedling; 4, leaf of two- to three-tiller stage; 5, sheath of two- to three-tiller stage; 6, root of two- to three-tiller stage; 7, panicle; 8, leaf of booting stage.
The PCR primers flanking the introns of the genes were RKb-3′race2 and RKb-2R for *Xa26*, and RKa-2L and RKa-22R for *RKa*. Genomic DNA (DNA) was used as control to distinguish PCR products from cDNA and genomic DNA.

the expression of *RKa* was higher in leaves, sheaths, and roots of seedling stage than in the tissues from other growth stages (Figure 2c). Thus, *Xa26* and *RKa* have different expression patterns.

The positions of the promoters relative to the coding regions predicted by programs TSSP, NNPP, and PROSCAN differed among the *Xa26* family members varying from upstream, to residing inside, of the coding sequences. The predicted promoter regions did not show significant sequence similarity among the members (data not shown). These differences may account for the distinct expression patterns of the *Xa26* family members.

Rice cultivars IRBB3 and Zhachanglong also carry Xa26

Comparing the map location of *Xa26* with previously mapped two *Xoo* resistance genes, *Xa3* carried by IRBB3 (*O. sativa* ssp. *indica*) and *Xa22(t)* carried by Zhachanglong (*O. sativa* ssp. *japonica*), it was speculated that *Xa3* and *Xa22(t)* are either tightly linked or allelic to *Xa26* (Lin *et al.*, 1996; Yang *et al.*, 2003; Yoshimura *et al.*, 1995). To determine whether IRBB3 and Zhachanglong have homologous sequences of *Xa26*, the expression of *Xa26*-like genes in Zhachanglong, IRBB3, and its susceptible near-isogenic parental line IR24 was examined using primers flanking the intron of *Xa26*. Expression profiling showed that the sizes of both RT-PCR products and the control PCR products

using total DNA as the templates from Zhachanglong and IRBB3 were the same as those from Minghui 63 (Figure 2b). Whereas, the relative sizes of both RT-PCR and control PCR products from IR24 were different from those of Minghui 63, Zhachanglong, and IRBB3. These results suggest that Zhachanglong and IRBB3 carry the homologs of *Xa26*.

To confirm the assertion that Zhachanglong and IRBB3 carry *Xa26*, DNA fragments containing the members of the *Xa26* gene family from Zhachanglong and IRBB3 were obtained using the following strategy. There were two *Spe*I restriction sites in the sequences of the BAC clone 3H8 flanking a region of about 19 kbp that contained both *Xa26* and its family member *RKa* (Figure 1a). Hybridization bands of the same size were produced when the genomic DNA from Minghui 63, Zhachanglong, and IRBB3 was digested with *Spe*I and hybridized with *RKa* as the probe (data not shown). To recover the 19-kbp fragment, total DNA of Zhachanglong and IRBB3 was digested to completion with *Spe*I and separated by electrophoresis. DNA fragments approximately 19 kbp in size were cut off the agarose gel, ligated to vector pCLD04541, and transformed into *Escherichia coli*, resulting in two mini-libraries. The libraries were screened by PCR amplification using primers (RKa-1L and RKa-1R) specific for *RKa*. About 3000 clones were screened and one positive clone was identified from each of the mini-libraries. The positive clones were sequenced, which revealed that the relative locations, transcription orientations, and sequences of the alleles of *Xa26* and *RKa* as well as their predicted promoter regions in Zhachanglong and IRBB3 were identical to those of Minghui 63. Thus, both Zhachanglong and IRBB3 carry *Xa26*.

To further determine the identities of *Xa3* and *Xa22(t)* in relation to *Xa26*, we compared the resistance of Minghui 63, IRBB3, and Zhachanglong by inoculating them with a set of differential *Xoo* strains (Table 3). The resistance spectra of the three cultivars appeared to be similar, but the resistance of Minghui 63 to almost all the *Xoo* strains examined was weaker than the other two lines. As *Xa26* was mapped precisely to the same chromosomal location of *Xa3* and *Xa22(t)* (also see Discussion), *Xa26*, *Xa3*, and *Xa22(t)* may be the same gene. One possible explanation for the lesion length differences observed among these lines is the influence of genetic backgrounds that are different in these genotypes.

Table 3 Comparison of the *Xoo* resistance of four varieties measured by lesion length[a]

Xoo strain	IRBB3 (*Xa3*)	Minghui 63 (*Xa26*)	Zhachanglong (*Xa22(t)*)	IR24 (susceptible)
PXO61	2.1 ± 1.2	8.1 ± 2.7	2.7 ± 1.3	22.4 ± 3.7
PXO86	3.0 ± 1.4	7.2 ± 3.7	4.0 ± 2.3	20.0 ± 2.2
PXO79	1.4 ± 0.6	5.3 ± 3.1	5.3 ± 3.1	24.8 ± 7.4
PXO112	4.4 ± 2.4	8.1 ± 1.8	2.0 ± 1.3	7.7 ± 3.8
PXO99	21.3 ± 4.1	26.4 ± 5.2	23.4 ± 6.4	26.5 ± 3.4
LN44		7.1 ± 3.3	5.4 ± 3.6	19.3 ± 5.2
JL691		1.5 ± 2.7	2.2 ± 3.8	11.4 ± 7.4

[a]Five to seven uppermost fully expanded leaves of each plant were inoculated at the booting stage. The lesion lengths (cm) were measured 21 days after inoculation.

Transgenic plants carrying Xa26 *had broader spectrum and higher level of resistance than the donor parent*

We also compared the resistance spectrum of transgenic plants carrying *Xa26* with that of the gene-donor parent Minghui 63 and recipient parent Mudanjiang 8 as indicated by lesion lengths at the booting stage, using 14 *Xoo* strains representing a wide range of bacterial blight strains (Table 4). The resistance spectrum of transgenic plants was much wider than Minghui 63 and IRBB3, both carrying *Xa26*. Minghui 63 and IRBB3 were susceptible or moderately susceptible to *Xoo* strains HB84-17, NX-42, LN85-57, PXO71, and PXO145, while the transgenic plants were highly resistant to all strains except to Zhe173 and PXO99 (Table 4). The resistance of transgenic plants to strains JL691, JS49-6, and PXO61 was also stronger than Minghui 63 and IRBB3.

To examine whether the resistance of the transgenic plants was influenced by copy number of the transgene, 13 randomly chosen resistant T_0 plants were examined by Southern hybridization analysis using a fragment of *Gus* gene as the probe. The results showed that one plant contained two copies of the gene, two contained three copies, and the remaining plants carried only one copy of the transgene. There was no relationship between the copy number and the level of resistance (Table 5). In addition, 80 T_1 plants of transgenic line Rb49 were inoculated with *Xoo* strain PXO71, a strain causing compatible reaction with the gene donor Minghui 63 (Table 2). Both the resistance and the transgene segregated in a 3 : 1 ratio, also suggesting that there was no relationship between copy number of the transgene and the enhanced resistance.

Minghui 63 was resistant to *Xoo* strain JL691 (Chen *et al.*, 2002), but susceptible to strains PXO61 and PXO86 at seedling (four-leaf) stage (unpublished data). To investigate whether the resistance spectrum of transgenic plants was also broadened at seedling stage, the resistant transgenic plants of T_2 generation were compared with Minghui 63, Mudanjiang 8, IRBB3, and Wase Aikoku 3 (*O. sativa* ssp. *japonica*) that is the *Xa3* donor for IRBB3 (Ogawa *et al.*, 1986c, 1988b, 1991), by inoculating with *Xoo* strains at the early seedling stage (Table 6). The seedlings of the transgenic plants were highly resistant to PXO61 and PXO86. Whereas, Minghui 63 and IRBB3 were susceptible to PXO86 at two-leaf stage, and IRBB3 was only moderately resistant to PXO61 and PXO86 at four-leaf stage. These results indicate that the transgenic plants also had a broader

Table 4 Resistance spectrum of *Xa26* measured by lesion length[a]

Strain	Group[b]	Mudanjiang 8	Transgenic	Minghui 63	IRBB3	IR24	Test[a]
JL691		7.7 ± 2.7	0.8 ± 0.7		2.2 ± 0.6		G
HB84-17	C2	18.7 ± 1.8	1.5 ± 0.5	11.3 ± 4.8	8.7 ± 2.7	13.6 ± 4.3	F
NX-42	C3	10.6 ± 1.7	0.7 ± 0.2		5.6 ± 1.2		G
Zhe173	C4	9.6 ± 2.8	7.4 ± 2.7	10.6 ± 3.3	5.8 ± 1.6	7.8 ± 2.3	F
GD1358	C5	4.0 ± 1.1	0.7 ± 0.4		1.1 ± 0.5		G
LN85-57	C6	17.2 ± 1.1	1.9 ± 0.7	9.5 ± 2.9	5.3 ± 1.9	14.3 ± 3.4	F
JS49-6	C7	21.5 ± 1.9	1.5 ± 0.9	8.7 ± 2.7	8.6 ± 2.3	16.9 ± 3.4	F
PXO61	Race 1	17.7 ± 2.9	0.8 ± 0.6	11.8 ± 4.1		17.7 ± 3.5	F
PXO86	Race 2	8.3 ± 1.7	0.7 ± 0.5		0.9 ± 0.1	6.4 ± 1.3	G
PXO79	Race 3	8.0 ± 0.9	1.0 ± 0.6		1.5 ± 0.9	8.3 ± 1.6	G
PXO71	Race 4	16.2 ± 3.4	1.1 ± 1.0	9.9 ± 3.3	3.8 ± 1.4	8.5 ± 2.5	F
PXO112	Race 5	16.4 ± 4.7	1.3 ± 0.8	8.0 ± 2.2	3.9 ± 1.0	10.5 ± 2.9	F
PXO99	Race 6	13.1 ± 2.7	12.3 ± 2.7		9.2 ± 2.6	8.2 ± 3.2	G
PXO145	Race 7	3.1 ± 0.8	1.6 ± 1.0	5.1 ± 1.5	3.7 ± 1.2	8.5 ± 5.1	F

[a]Three to seven uppermost fully expanded leaves of each plant were inoculated at the booting stage. The lesion lengths (cm) were measured 21 days after inoculation. For the transplants inoculated in the greenhouse (G), each data point was obtained from one resistant T_0 plant and one resistant T_1 plant that was from a transgenic line other than the T_0 plant inoculated with the same *Xoo* strain. For the transgenic plants inoculated in the field (F), each data point was collected from three to five resistant T_1 plants from the same transgenic line. Totally 12 transgenic lines including Rb17, Rb18, and Rb22 listed in Table 2 were used for the inoculation.
[b]Racial groups of *Xoo*: C2–C7, Chinese groups; race 1 to race 7, Philippine groups.

spectrum of resistance than Minghui 63 and IRBB3 at the seedling stage.

Discussion

The function of the XA26 protein

The predicted encoding product of *Xa26* has a typical structure of LRR receptor kinase-like protein, the extracellular domain of LRR, the transmembrane region, and the cytoplasmic domain of protein kinase. Among the reported plant LRR receptor kinase-like proteins, the XA26 protein has the highest sequence homology with rice bacterial blight resistance protein XA21 (Song *et al.*, 1995). *Xa21* and *Xa26* are the only *R* genes confirmed so far to encode LRR receptor kinase-like proteins, although many *R* genes have been isolated from various plant species (Hulbert *et al.*, 2001). Although the encoding products of the two genes share 53% sequence similarity (*E*-value = e^{-135}), the resistance spectra mediated by *Xa26* and *Xa21* are different (Wang *et al.*, 1996). The distinguishable structural difference between the two genes is that *Xa26* encodes 26 LRRs while *Xa21* has 23 LRRs (Song *et al.*, 1995). The LRR domain of resistance proteins of plants is suggested to directly interact with the product of the avirulence gene or its indirect product. It is known that the race-specificity of XA21 is determined by its LRR domain (Wang *et al.*, 1998). The xxLxLxx motif in each LRR is predicted to form a short β-strand/β-turn structure, in which the five interstitial residues (x) are predicted to be solvent exposed (Jones and Jones, 1997). The short β-strand/β-turn regions are predicted to form a parallel β-sheet, which could create a surface for ligand interactions (Kobe and Deisenhofer, 1995). About 87% of the predicted solvent exposed amino acids in XA26 and XA21 proteins are different (Figure S2). Thus, the structural difference and the variants of solvent-exposed amino acids in the LRR domains may be the causes for the difference in resistance spectra conferred by the two genes.

Developmentally controlled disease resistance has been observed in many plant-pathogen systems (Century *et al.*, 1999; Panter *et al.*, 2002). Full disease resistance usually occurs at adult stage in these systems. *Xa21*-mediated

Table 5 Resistance of transgenic plants and copy number of the transgene[a]

T_0 plant	Lesion length (cm)	Copy number
Rb1	2.3 ± 1.96	1
Rb5	0.2 ± 0.00	3
Rb6	0.7 ± 0.83	1
Rb19	1.4 ± 0.93	1
Rb23	0.3 ± 0.18	1
Rb24	0.5 ± 0.42	1
Rb26	0.9 ± 0.81	1
Rb29	0.3 ± 0.00	3
Rb35	0.2 ± 0.12	1
Rb37	0.8 ± 0.56	1
Rb63	1.5 ± 1.33	1
Rb68	1.6 ± 1.14	2
Rb75	0.3 ± 0.15	1

[a]Two to six uppermost fully expanded leaves of each T_0 plant were inoculated with *Xoo* strain PXO86 at the booting stage. The lesion lengths of cultivars IRBB3 (carrying *Xa26*) and Mudanjiang 8 were 3.2 ± 0.87 and 8.0 ± 0.40 cm, respectively.

Table 6 Resistance of different rice materials at seedling stage[a]

Material	*R* gene	Two-leaf stage (PXO86)	Four-leaf stage	
			PXO61	PXO86
Mudanjiang 8	Susceptible	6.0 ± 2.0	12.7 ± 2.6	15.1 ± 2.6
Transgenic plant	*Xa26*	0.6 ± 0.4	0.9 ± 0.5	1.0 ± 0.3
IRBB3	*Xa26* (*Xa3*)	5.1 ± 2.3	3.8 ± 2.2	4.4 ± 1.9
Wase Aikoku 3	*Xa3*	0.5 ± 0.3		
Minghui 63	*Xa26*	6.1 ± 1.8		

[a]The uppermost fully expended leaves of each plant were inoculated in the greenhouse. The lesion length (cm) was measured 15 days after inoculation and averaged over 7–15 plants.

resistance increases progressively from the susceptible juvenile stage to full resistance at later adult stage. However, *Xa21* is expressed in leaves at both susceptible and resistant stages, indicating that developmental regulation of *Xa21*-mediated resistance is either controlled post-transcriptionally or by other factors (Century *et al.*, 1999). The present results show that *Xa26* gene was also expressed constitutively in leaf tissue and its expression level was not influenced by pathogen infection and wounding. Whereas, *Xa26*-mediated resistance was detected from very early seedling stage to adult stages, suggesting that the resistance conferred by *Xa26* is not developmentally regulated.

The relationship of Xa3*,* Xa22(t)*, and* Xa26

IRBB3, a near-isogenic line of IR24 (Ogawa *et al.*, 1986c, 1988b, 1991), presumably contains only *Xa3* for bacterial blight resistance. *Xa3* is also mapped to the long arm of chromosome 11 and tightly linked or allelic to another bacterial blight resistance gene *Xa4* (Yoshimura *et al.*, 1995). Fine genetic mapping of *Xa4* revealed that *Xa4* and *Xa26* are tightly linked (Sun *et al.*, 2003; Yang *et al.*, 2003). However, physical mapping of *Xa4* and sequence analysis of the BAC clone containing *Xa4* gene suggests that *Xa4* is a member of the *Xa26* family, but not allelic with *Xa26* (X. Sun *et al.*, unpublished data). Analysis of 280 highly susceptible and 260 randomly chosen F_2 plants from a cross between IRBB3 and IR24 for resistance to *Xoo* strain PXO86 showed that the resistance co-segregated with *Xa26*, while recombination was detected between resistance and *RKa* (Y. Xiang *et al.*, unpublished data). The present results confirmed the existence of *Xa26* and *RKa* in IRBB3, hence providing evidence strongly suggesting that *Xa3* and *Xa26* are the same gene.

The *Xa22(t)* gene is identified in Zhachanglong, a *japonica* variety showing resistance against a wide range of *Xoo* strains, and also mapped to the long arm of rice chromosome 11 (Lin *et al.*, 1996). Fine genetic mapping of *Xa22(t)* revealed that this gene is also tightly linked or allelic to *Xa26* (Wang, 1999; Yang *et al.*, 2003). The confirmation of the existence of *Xa26* gene in Zhachanglong in the present study suggests that *Xa22(t)* and *Xa26* may also be the same gene. Therefore, it is highly likely that *Xa3*, *Xa22(t)*, and *Xa26* are the same gene, although final resolution of the relationships of these genes awaits the detailed physical mapping of *Xa3* and *Xa22(t)*.

Effects of genetic backgrounds on resistance mediated by Xa26

Lesion length comparisons revealed that the transgenic plants were more resistant to *Xoo*, in terms of both spectrum and level, than to Minghui 63 and IRBB3 at both adult and seedling stages (Tables 2, 4, and 6). Enhanced resistance was also reported in *Xa21* transgenic lines as compared to the gene-donor line (Song *et al.*, 1995; Wang *et al.*, 1996). The increased resistance of *Xa21* transgenic lines was ascribed to multiple copies of the transgene because of transformation by particle bombardment (Wang *et al.*, 1996). In the case of *Xa26*, we showed that the enhanced resistance was not associated with copy number of the transgenes. It should be noted that the enhanced resistance was observed even in the T_0 generation of transgenic plants that were hemizygous for *Xa26*, also substantiating that the enhanced resistance is unlikely the results of multiple copies of the transgene.

Interestingly, both Minghui 63 and IRBB3 are of *indica* genetic background, and the transgene in Mudanjiang 8 is in a *japonica* genetic background. These results indicate that genetic background may influence *Xa26*-mediated resistance. This notion will be further enhanced if *Xa26*, *Xa3*, and *Xa22(t)* are indeed the same gene. The effect of genetic background on *R* gene has also been observed in *Arabidopsis*. An allele of *Arabidopsis R* gene *RPS2*, encoding a protein with an 11 aa difference from RPS2, does not function in the Po-1 genetic background, but can confer full resistance to *P. syringae* expressing *avrRpt2* in the Columbia (Col-0) background (Banerjee *et al.*, 2001).

Supporting evidence may also be obtained by comparing the resistance of different rice cultivars that carry *Xa26* gene. IRBB3 is suggested to have only *Xa3* for *Xoo* resistance (Ogawa *et al.*, 1991), which is likely to be *Xa26*

according to the above discussions. Whereas, Minghui 63 carries another major gene for *Xoo* resistance, *Xa25(t)*, in addition to *Xa26* (Chen *et al.*, 2002). However, IRBB3 showed better resistance than Minghui 63 to all the *Xoo* strains that we tested (Tables 3 and 4). In this connection, it is interesting to note that *Xa3* is the most misapprehended resistance gene in the literature of rice bacterial blight studies. This gene has been named *Xa3*, *Xa4b*, *Xa6*, and *Xa9*, largely because it behaved very differently in different genetic backgrounds and even displayed dominance reversal in one case (Ogawa *et al.*, 1986a,b,c, 1988a; Sidhu and Khush, 1978). It is also interesting to note that the *japonica* variety Wase Aikokus 3, the *Xa3* gene donor for IRBB3, was highly resistant to *Xoo* strain PXO86 at the seedling stage, while IRBB3 and Minghui 63 were susceptible (Table 6). Moreover, *Xoo*-resistance spectrum of Zhachanglong that carries *Xa22(t)* (also highly likely to be *Xa26*) is much broader than Minghui 63 and IRBB3 (Lin *et al.*, 1996; Yang *et al.*, 2003), although part of the difference conferred may be ascribed to the presence of another *Xoo*-resistance gene in Zhachanglong (Wang, 1999). All these results indicate that genetic backgrounds may play important roles in the resistance conferred by *Xa26*. Comparison of the performance of the gene in the backgrounds of Mudanjiang 8, Zhachanglong, and Wase Aikoku 3 against Minghui 63 and IRBB3 seems to suggest that *Xa26* performs better in a *japonica* background than in an *indica* background. These results may have some generality for the study of plant disease resistance, implying that the observed level and spectrum of resistance activity apparently conferred by a resistance gene are not only determined by the gene itself but also largely dependent on the genetic background of the host carrying the gene. Identification of the factor(s) regulating the *R* gene-mediated resistance in different genetic backgrounds will help understanding the molecular mechanisms of resistance and also help formulating strategies for breeding programs.

Experimental procedures

DNA sequencing

A shotgun approach was used to determine the nucleotide sequence of BAC clone 3H8. The M13 universal forward and reverse primers and the BigDye Terminator Cycle Sequencing v2.0 (Applied Biosystems, Foster City, CA, USA) were used for sequencing. Sequence contigs were assembled using the computer program SEQUENCHER 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

Gene expression analysis

Total RNA was isolated from rice tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Gene expression in each of the tissues was analyzed three times by RT-PCR. The RT-PCR was carried out as a two-step reaction (Zhou *et al.*, 2002). In brief, the RT was performed in a 20 μl volume containing 3 μg of total RNA, 200 ng oligo $(dT)_{15}$ primer, 200 U Molony Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen), 1× first strand buffer, 2 mM DTT and 1 mM deoxynucleoside triphosphate (dNTP) at 42°C for 1.5 h. The RT was terminated by incubating at 70°C for 10 min. An aliquot (0.5–2 μl) of the reaction mixture was used for PCR. The primers for the *Xa26* gene were RKb-3′race2 (5′-TGGTCAAATACCGGAAGGAG-3′), RKb-2R (5′-CAGTCCACCACATGGACAAG-3′), RKb-3′race1 (5′-CCATCCCAAACTACTTGGCTA-3′), RKb-R (5′-GCTTCCCTTGTTCTGAGTGC-3′), RKb-4F (5′-AGCGATGATAGCATGTTGGG-3′), and RKb-3R (5′-TCAGTGTCAAGACCACATCG-3′). Other primers used included: RKa-2L (5′-CTGCATGGTCAGATACCAAAAG-3′), RKa-22R (5′-TCGAGTAGCATGATCCCGTA-3′), RKa-1L (5′-GGTTAGTCACAAGTGGGAAAGG-3′), and RKa-1R (5′-AAGATGAAATATGCTCGGTGGT-3′) for *RKa*; RKc-F (5′-TGTTTCGAGTGGCATACAGC-3′) and RKc-R (5′-ATGAGCCGAGCAATGATACC-3′) for *RKc*; and LRR-1F (5′-GCAAAGCACCCTCGAATAAG-3′) and LRR-1R (5′- ATCACAAACCTCACGGGTTC-3′) for *RKd*. The locations of the primers for *Xa26* family members are shown in Figure S1. The actin primers (Yoshimura *et al.*, 1998) were used as standard in all the RT-PCR assays.

Rice transformation

Bacterial artificial chromosome clone 3H8 containing *Xa26* was digested with different restriction enzymes. Two fragments resulting from the digestion with *Dra*I, 6.5 and 7.5 kbp in length, contained, respectively, *RKa* and *Xa26* with their native promoters. A 9.6-kbp fragment digested with *Bgl*II contained *RKc* and its promoter. The fragments containing *Xa26* and *RKa* were ligated with the vector pCAMBIA 1301 digested with restriction enzyme *Sma*I, and the fragment containing *RKc* was ligated with the vector treated with *Bam*HI.

The constructs containing *Xa26*, *RKa*, and *RKc* were transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium*-mediated transformation was performed according to Hiei *et al.* (1994). The copy number of *Xa26* in transgenic plants was determined by DNA gel blot analysis with probes amplified using *Gus*-specific primers: GusF (5′-CCAGGCAGTTTTAACGATCAGTTCGC-3′) and GusR (5′-GAGTGAAGATCCCTTTCTTGTTACC-3′).

Pathogen inoculation and disease scoring

A number of *Xoo* strains from China and Philippines was used for inoculation. Plants were inoculated at either seedling or booting stages in the field during summer or in a greenhouse by leaf-clipping method (Kauffman *et al.*, 1973). For inoculation in greenhouse, the temperature was maintained at 31°C in daytime and 25°C at night with 12-h photoperiod. The bacterial inoculum was prepared as described previously by Lin *et al.* (1996). Mock-inoculated (control) plants were treated under the same condition except that pathogen inoculum was replaced with water. The disease was scored by measuring the lesion length (in cm) at 2 weeks after inoculation for seedlings and 2–3 weeks after inoculation for adult plants.

Gene structure analysis

The transcripts of *Xa26* and *RKa* were examined by RT-PCR using primers RKb-3′race2 and RKb-2R flanking the predicted intron of *Xa26* and RKa-2L and RKa-22R flanking the predicted intron of *RKa*.

The 3′ and 5′ untranslated regions (UTRs) of *Xa26* gene were analyzed by 3′- and 5′-RACE methods using the 3′-Full RACE Core Set and 5′-Full RACE Core Set kits (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocols, except that the Avian Myeloblastosis Virus (AMV) reverse transcriptase in the kits was replaced with SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). The first and second rounds PCR primers for 3′-RACE were RKb-3′race1 and RKb-3′race2, respectively. The RT primer of 5′-RACE was 5′-ATCAACCGGCA-3′. The primers RKb-5′raceA1 (5′-GCGTGTCCATACCTCCAAGT-3′) and RKb-5′raceS1 (5′-CGTA-GAACTGGGAGGCTGAA-3′) were used for the first round of PCR, and primers RKb-5′raceA2 (5′-CCAATCTTGATGCCATCTCC-3′) and RKb-5′raceS2 (5′-CCGCCCCAGTTAAGTTATTG-3′) were used for second round of PCR in 5′-RACE. The products of RT-PCR and 3′- and 5′-RACEs were cloned using pGEM-T Easy Vector Systems (Promega, Madison, WI, USA).

DNA and protein sequence analysis

DNA sequence similarity analysis was performed using BLASTN and BLASTX (Altschul *et al.*, 1997). Gene prediction programs used were GENSCAN (Burge and Karlin, 1997) and FGENESH (http://www.softberry.com). Promoter regions of the *Xa26* family members were analyzed with the promoter prediction programs TSSP (http://www.softberry.com/berry.phtml), NNPP (http://www.fruitfly.org/seq_tools/promoter.html), and PROSCAN (http://bimas.dcrt.nih.gov/molbio/proscan). Protein sequence similarity analysis was performed using BLASTP program (Altschul *et al.*, 1997). The IPSORT (http://hypothesiscreator.net/iPSORT) was used for prediction of signal peptide and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) for prediction of the transmembrane region. The putative *N*-linked glycosylation sites of the XA26 protein were analyzed using NETNGLYC (http://www.cbs.dtu.dk/services/NetNGlyc).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1976/TPJ1976sm.htm

Figure S1. Nucleotide acid sequence comparison of the encoding regions of *Xa26* gene family members and the *Xa21* gene.

The sequence of *Xa26* is presented. The identical nucleotide acids of other genes are indicated by dots and gaps are shown with dashes. The positions of primers used in this study are indicated with black shadings and arrows. The primer RKa-1L is located 214 bp upstream of translation start codon of *RKa* gene. Two frameshift sites and one nonsense mutant site of *RKd* are shaded with gray color.

Figure S2. Sequence comparison of the LRR domains of XA26 and XA21 proteins.

The LRR sequence of XA26 protein is presented. The identical amino acid residues of XA21 protein are indicated by dots and gaps are shown with dashes. The solid black shaded residues indicate the solvent exposing amino acid. The LRR of XA26 and XA21 proteins are numbered, respectively. The borders of each LRR are shown by asterisk (*).

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