Theor Appl Genet (2009) 119:231–239
DOI 10.1007/s00122-009-1032-3

ORIGINAL PAPER

Dissection of the factors affecting development-controlled and race-specific disease resistance conferred by leucine-rich repeat receptor kinase-type *R* genes in rice

Jing Zhao · Jing Fu · Xianghua Li · Caiguo Xu · Shiping Wang

Received: 2 October 2008 / Accepted: 5 April 2009 / Published online: 24 April 2009

Abstract Development-controlled resistance and resistance specificity frequently restrict the application of a disease resistance (*R*) gene in crop breeding programs. *Xa3/Xa26* and *Xa21*, encoding leucine-rich repeat (LRR)-kinase type plasma membrane proteins, mediate race-specific resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes bacterial blight, one of the most devastating rice diseases. Plants carrying *Xa3/Xa26* and plants carrying *Xa21* have different resistance spectra and the functions of the two *R* genes are regulated by developmental stage. Four chimeric genes encoding proteins consisting of different parts of XA3/XA26 and XA21 were constructed by domain swapping and transformed into a susceptible rice variety. The resistance spectra and development-regulated resistance of the transgenic plants carrying *Xa3/Xa26*, *Xa21*, or chimeric gene to different *Xoo* strains were analyzed in the same genetic background. The results suggest that the gradually increased expression of *Xa3/Xa26* and *Xa21* plays an important role in the progressively enhanced *Xoo* resistance during rice development. In addition, the LRR domains of XA3/XA26 and XA21 are important determinants of race-specific recognition during rice–*Xoo* interaction, but juxtamembrane regions of the two R proteins also appear to contribute to resistance specificity.

Communicated by T. Tai.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-1032-3) contains supplementary material, which is available to authorized users.

J. Zhao · J. Fu · X. Li · C. Xu · S. Wang (✉)
National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, 430070 Wuhan, China
e-mail: swang@mail.hzau.edu.cn

Introduction

Rice is a staple food worldwide, especially in Asia. Bacteria blight, which is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases restricting rice production. Rice resistance to *Xoo* is regulated by disease resistance (*R*) genes and resistance quantitative trait loci (QTLs). Six *R* genes, *Xa1*, *Xa3/Xa26*, *xa5*, *xa13*, *Xa21*, and *Xa27* (Song et al. 1995; Yoshimura et al. 1998; Iyer and McCouch 2004; Sun et al. 2004; Gu et al. 2005; Chu et al. 2006; Xiang et al. 2006), and four resistance QTL genes, *OsDR6*, *OsDR8*, *OsWRKY13*, and *GH3-8* (Wang et al. 2006; Qiu et al. 2007; Yuan et al. 2007; Ding et al. 2008; Hu et al. 2008), conferring resistance to *Xoo* have been characterized. Although much progress has been made in understanding the details of the interaction between rice and *Xoo*, the molecular mechanisms of rice resistance to *Xoo* remain unclear.

One question yet to be answered is how rice plants regulate development-controlled resistance to *Xoo*. *Xa3/Xa26* and *Xa21* encode leucine-rich repeat (LRR) receptor kinase-type proteins (Song et al. 1995; Sun et al. 2004), which are the only two characterized plant LRR receptor kinase R proteins mediating race-specific resistance. The activity of *Xa21* is developmentally controlled. *Xa21*-mediated resistance to Philippine *Xoo* strain PXO99 increases progressively from the susceptible juvenile stage to full resistance at the adult stage (Century et al. 1999). *Xa3/Xa26*-mediated resistance is developmentally controlled only for some strains of *Xoo*. A rice line carrying *Xa3/Xa26* was susceptible at the seedling stage and became moderately resistant at adult stage to Philippine *Xoo* strain PXO61 (Cao et al. 2007a), but this line was resistant to Chinese *Xoo* strain JL691 at both seedling and adult stages (Chen et al. 2002). Some other *R* genes, such as the uncharacterized *Xa4*, can

mediate full resistance to *Xoo* at both the seedling and adult stages (Ogawa et al. 1986). Xu et al. (2006) found that the protein stability of XA21 contributed to the resistance and XA21 autophosphorylation facilitated its stability. The degradation of XA21 was developmentally regulated; this protein, however, was more stable at the seedling stage, in which XA21 did not confer resistance (Xu et al. 2006). These results suggest that XA21 stability may not be the critical factor for the development-controlled resistance. Our recent study revealed that development-controlled *Xa3/Xa26* activity is associated with its expression level (Cao et al. 2007a). The expression level of *Xa3/Xa26* is very low at the two-leaf stage, gradually increases with development, and reaches the highest level at the maximum-tillering to booting stage. Transgenic rice plants with a high level of *Xa3/Xa26* transcripts at both seedling and adult stages showed stable full resistance to *Xoo* throughout their development. However, we do not know whether *Xoo* resistance mediated by other *R* genes, whose functions are also regulated by development, is also gene dosage-dependent.

Another question is how rice regulates race-specific resistance to *Xoo*. Although both *Xa3/Xa26* and *Xa21* encode similar types of proteins, the resistance spectra mediated by the two genes are different. For example, a rice plant carrying *Xa21* is resistant to *Xoo* strain PXO99 and susceptible to Philippine *Xoo* strain PXO341 (Song et al. 1995; Narayanan et al. 2002), while plant carrying *Xa3/Xa26* is susceptible to PXO99 and resistant to PXO341 (Sun et al. 2004; Shiping Wang, unpublished data). The recognition specificity determines the resistance spectrum conferred by an *R* gene. Although the amino acid sequence, especially the LRR sequence of LRR-containing R proteins, is the major determinant of pathogen recognition (Dangl and Jones 2001), accumulating data indicate that other regions of LRR-containing R proteins, such as the Toll/interleukin-1 receptor homology region and the region between signal peptide and LRR domain, also influence resistance specificity (Ellis et al. 1999; Van der Hoorn et al. 2001). Our previous study indicated that the kinase domain appeared to influence *Xa3/Xa26*-mediated *Xoo* recognition. Substitution of the kinase domain of XA3/XA26 with that of its family member MRKa compromised rice resistance to *Xoo* (Cao et al. 2007b).

To further investigate these questions, we used the interactions between XA3/XA26 and *Xoo* and between XA21 and *Xoo* as models. The results suggest that, like *Xa3/Xa26*, development-controlled *Xa21* function is also related to its expression level. XA3/XA26 and XA21 each contain an extracellular LRR domain, a transmembrane (TM) region, and a cytoplasmic kinase domain. Domain swapping between XA3/XA26 and XA21 showed that in addition to the LRR domain, the juxtamembrane regions of XA3/XA26 and XA21 also affected pathogen recognition conferred by the two R proteins.

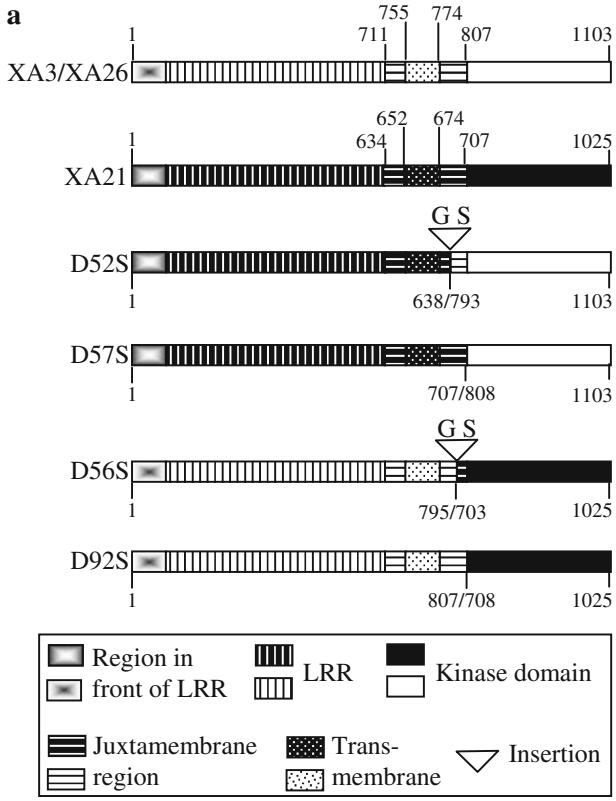
Materials and methods

Construction of chimeric genes and plant transformation

Four chimeric genes (*D52S*, *D56S*, *D57S*, and *D92S*) consisting of different fragments of *Xa3/Xa26* and *Xa21* were constructed. DNA fragments encoding different domains of XA3/XA26 and XA21 were obtained by PCR amplification using gene-specific primers (Supplementary Table 1). *D52S* and *D56S* were constructed by ligation of restriction endonuclease *Bam*HI digested fragments, while *D57S* and *D92S* were obtained by overlap extension PCR (Horton et al. 1989). *D52S* consists of the sequence encoding the extracellular LRR domain, TM region, and partial cytoplasmic juxtamembrane region of XA21 (amino acids 1 to 683), an adaptor (GGATCC) insertion encoding glycine (G) and serine (S) that was used as the cleavage site of *Bam*HI in constructing the chimeric gene, and the sequence encoding partial cytoplasmic juxtamembrane region and intact kinase domain of XA3/XA26 (amino acids 793–1,103; Fig. 1). *D56S* consists of the sequence encoding the extracellular LRR, TM region, and partial cytoplasmic juxtamembrane region of XA3/XA26 (amino acids 1–795), an adaptor insertion encoding G and S, and the sequence encoding partial cytoplasmic juxtamembrane region and intact kinase domain of XA21 (amino acids 703–1,025). *D57S* consists of the sequence encoding the LRR domain, TM region, and juxtamembrane region of XA21 (amino acids 1–707) and the sequence encoding the kinase domain of XA3/XA26 (amino acids 808–1,103). *D92S* consists of the sequence encoding the LRR domain, TM region, and juxtamembrane region of XA3/XA26 (amino acids 1–807) and the sequence encoding the kinase domain of XA21 (amino acids 708–1,025). These genes were ligated into the transformation vector pS1301 (Cao et al. 2007a), which was modified by insertion of a cauliflower mosaic virus 35*S* promoter and a nopaline synthase polyadenylation signal terminator into the multiple cloning sites of vector pCAMBIA1301 (Supplementary Fig. 1). A 9.9-kb DNA fragment (including 2,204 nt upstream of translation start codon of *Xa21*, the coding region of *Xa21*, and 3787 nt downstream of translation stop codon of *Xa21*) containing *Xa21* and its native promoter derived from clone pC822 (Song et al. 1995) was ligated with vector pCAMBIA1301. Agrobacterium-mediated transformation was performed according to the method of Lin and Zhang (2005).

Pathogen inoculation

To evaluate the resistant spectra to bacterial blight disease, plants were inoculated with different Philippine *Xoo*



b

```
XA26: RKKANHQNTSAGKADLISHQLLSYHELLRATDD
XA21: TWHKRTKKGAPSRTSMKGHPLVSYSQLVKATDG
D52S: TWHKRTKKGAGS------HQLLSYHELLRATDD
D57S: TWHKRTKKGAPSRTSMKGHPLVSYSQLVKATDG
D56S: RKKANHQNTSAGKADLISHQLGS-----KATDG
D92S: RKKANHQNTSAGKADLISHQLLSYHELLRATDD
```

Fig. 1 The chimeric proteins from the recombination of XA21 and XA3/XA26. **a** Schematic diagram of the proteins encoded by the chimeric genes *D52S*, *D57S*, *D56S*, and *D92S*. The *numbers* indicate the amino acid position of each structure in the native protein. **b** The sequences of the cytoplasmic juxtamembrane regions located at the C-terminals of the transmembrane motifs in different proteins. The adaptor insertion encoding glycine (G) and serine (S) in D52S and D56S is shown in *italic*. Three autophosphorylation amino acid residues, Ser686, Thr688, and Ser689 in XA21 (Xu et al. 2006), are *underlined*

strains, PXO61 (race 1), PXO79 (race 3), PXO71 (race 4), PXO99 (race 6), PXO280 (race 8), and PXO341 (race 10), and Chinese strains JL691 and KS-1-21 at the booting stage by the leaf clipping method (Sun et al. 2004). Disease was scored by measuring the percent lesion area (lesion length/leaf length) at 2 weeks after inoculation. To examine the influence of developmental stage on the function of *Xa21* and *Xa3/Xa26*, different rice lines were grown with staggered planting so that *Xoo* inoculation could be carried out at designated developmental stages at the same time.

DNA gel blot analysis

Rice total DNA was digested with *Hin*dIII, separated by electrophoresis on 0.8% agarose gels, and blotted onto nylon membranes. The DNA fragment of hygromycin phosphotransferase gene amplified using gene-specific primers (Supplementary Table 1) was used as a hybridization probe to detect the copy number of transgenes.

RNA gel blot and quantitative reverse transcription (qRT)-PCR analyses

Aliquots (20 μg) of total RNA were used for RNA gel blot analysis (Chu et al. 2004). The 705-, 723-, 543-, and 534-bp probes of *D52S*, *D57S*, *D56S*, and *D92S*, respectively, amplified using primers flanking the chimeric sites of the genes, were used as hybridization probes (Supplementary Table 1). The qRT-PCR was conducted as described by Cao et al. (2007a). PCR primers for qRT-PCR are listed in Supplementary Table 1. The expression level of the rice actin gene (GenBank accession number X15865) was used to standardize the RNA sample for each qRT-PCR. Each qRT-PCR assay was repeated at least twice, with each repetition having three replicates.

Results

Plants carrying *D56S* or *D57S* confer resistance to *Xoo*

The four chimeric genes, *D52S*, *D56S*, *D57S*, and *D92S* (Fig. 1), driven by the constitutive promoter 35*S* were transferred separately into susceptible rice cultivar Mudanjiang 8 (*Oryza sativa* ssp. *japonica*), and 38 (D52SM), 39 (D56SM), 67 (D57SM), and 28 (D92SM) independent transformants carrying *D52S*, *D56S*, *D57S*, and *D92S*, respectively, were generated. For all transgenic rice plants overexpressing *D52S* (D52SM) and *D92S* (D92SM), no resistance was observed compared with Mudanjiang 8 when inoculated with *Xoo* strain PXO61 or PXO99. In contrast, a significant reduction in lesion area was observed in transgenic plants carrying *D56S* (D56SM) and *D57S* (D57SM) after *Xoo* infection. Twenty-five of the 39 T0 plants carrying *D56S* and 15 of the 34 T0 plants carrying *D57S* showed enhanced resistance to PXO61, with average lesion areas of $20.2 \pm 12.2\%$ and $19.1 \pm 11.0\%$, respectively, as compared to $70.5 \pm 9.4\%$ and $57.9 \pm 11.3\%$ for the control Mudanjiang 8 (Supplementary Table 2). Eleven of the 27 positive T0 plants carrying *D57S* were highly resistant to PXO99, with average lesion area of $14.6 \pm 2.6\%$ compared to $92.6 \pm 9.9\%$ for Mudanjiang 8 and $15.3 \pm 2.7\%$ for *Xa21* donor IRBB21 (Supplementary Table 2). Amplifications of *GUS* by PCR

were performed to confirm that all resistant plants carried the transformation construct (Supplementary Table 2).

T1 families derived from two of the resistant T0 plants carrying one copy of *D56S* or *D57S* were further examined individually for resistance by inoculating with PXO61 and for the presence of the transgene by gene expression and PCR analyses at the booting stage. The resistance cosegregated with *D56S* or *D57S* in the T1 families (Fig. 2; Supplementary Table 2), indicating that the resistance was due to the presence of *D56S* or *D57S*. The resistance of these transgenic plants was heritable. The homozygous T2 plants also showed enhanced resistance (Fig. 3a). Two homozygous lines, D56SM21-7 and D57SM5-5 carrying *D56S* and *D57S*, respectively, were subject to bacterial growth analysis. The growth rates of PXO61 on these transgenic plants at the booting stage were approximately 100-fold lower than that on wild-type Mudanjiang 8 at 14 days after inoculation (Fig. 3b).

We transferred *Xa21* and its native promoter into Mudanjiang 8 as a control. Fifteen independent positive transformants (D49OM) were generated. All these positive T0 plants showed enhanced resistance to *Xoo* strain PXO99, with average lesion area of 11.6 ± 2.9% compared to 49.9 ± 3.9% for the control of Mudanjiang 8 (Supplementary Table 2). The homozygous line D49OM6 carrying a single copy of *Xa21* was used for further analysis.

Xa3/Xa26, *Xa21* and their chimeric genes have different resistance activities

To determine the influence of gene structure on the resistance specificity conferred by *Xa3/Xa26* and *Xa21*, transgenic lines D49OM6 carrying *Xa21* and Rb49 carrying a single copy of *Xa3/Xa26* with the background of Mudanjiang 8 (Sun et al. 2004; Cao et al. 2007a) were analyzed together with the transgenic lines carrying chimeric genes. These plants were inoculated with different *Xoo* strains; PXO99 and PXO341 are known to induce different resistant or susceptible reactions in the rice varieties carrying *Xa3/Xa26* and *Xa21*, respectively. The results showed that transgenic line D49OM6 carrying *Xa21* was resistant to all the *Xoo* strains examined except PXO341 and the resistance spectrum of D49OM6 was the same as rice line IRBB21, the donor of *Xa21* (Table 1). Transgenic line Rb49 carrying *Xa3/Xa26* was resistant to all the strains except PXO99, PXO280, and KS-1-21, which was same as at least one of the two rice lines (IRBB3 and Minghui 63) carrying the same *R* gene. Proteins D57S and XA21 only differ in the kinase domain (Fig. 1). Transgenic line D57SM5 carrying *D57S* showed the same resistance spectrum as D49OM6 carrying *Xa21*, but the lesion areas of D57SM5 caused by different *Xoo* strains were 1.5- to 7.1-fold larger than those of D49OM6 (Table 1). Proteins D52S and D57S differed only by 14 amino acids in the juxtamembrane region

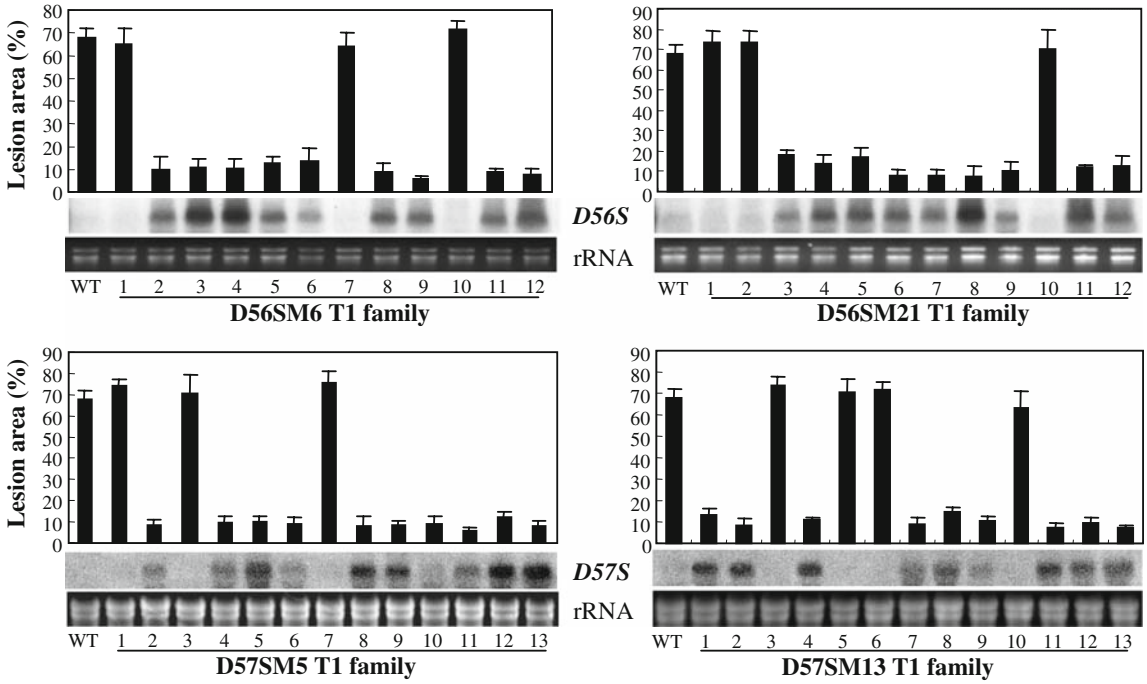


Fig. 2 Enhanced resistance to *Xoo* strain PXO61 cosegregated with overexpression of *D56S* in T1 families D56SM6 and D56SM21 or with overexpression of *D57S* in T1 families D57SM5 and D57SM13. Bars represent mean (three replicates) ± standard deviation. WT, wild type Mudanjiang 8

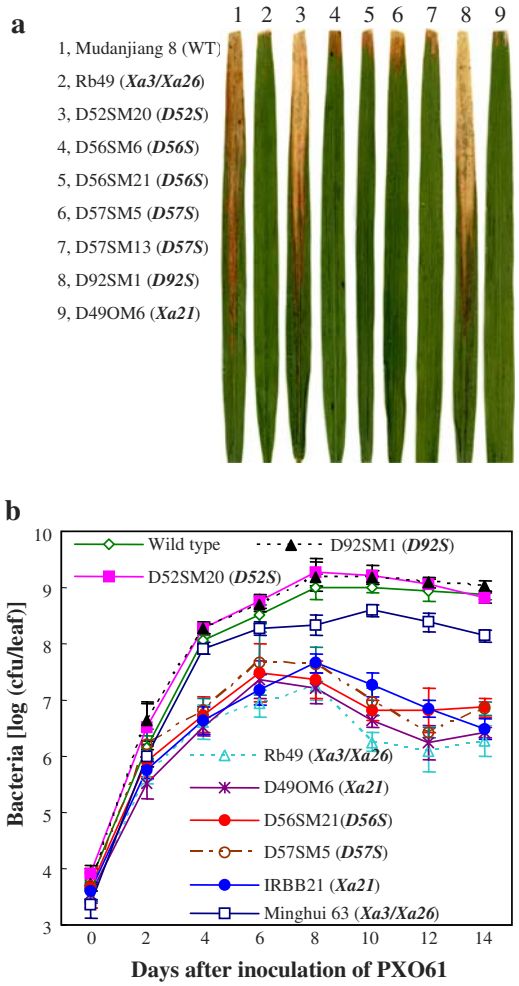


Fig. 3 Performance of transgenic plants carrying *Xa3/Xa26*, *Xa21*, or chimeric genes. All the transgenic plants are homozygous lines in the genetic background of wild-type (WT) Mudanjiang 8. **a** Leaves from plants at 14 days after inoculation with *Xoo* strain PXO61. **b** Growth of PXO61 in leaves of the different rice lines at the booting stage. The bacterial population was determined from three leaves at each time point by counting colony-forming units (cfu; Sun et al. 2004). Each point represents mean (three replicates) ± standard deviation

(Fig. 1). However, the transgenic line D52SM20 overexpressing *D52S* was susceptible to all the *Xoo* strains. These results suggest that the LRR domain is an important determinant of race-specific recognition conferred by XA21, but the juxtamembrane region and kinase domain also influence the function of XA21.

Transgenic line D56SM6 carrying *D56S* showed a resistance spectrum similar to that of Rb49 carrying *Xa3/Xa26* (Table 1). Rb49 was highly resistant to PXO61, PXO79, PXO71, and JL691 and susceptible to PXO99 and KS-1-21. Interestingly, D56SM6 showed slightly compromised resistance to PXO61, PXO79, PXO71, and JL691 with 1.2- to 10.6-fold larger lesion areas and enhanced resistance to PXO99 and KS-1-21 with 40 and 56% smaller lesion area as compared to Rb49. D56S and XA3/XA26 differ in the kinase domain and eight amino acid residues of the juxtamembrane region (Fig. 1). Transgenic line D92SM1 overexpressing *D92S* was susceptible to all the *Xoo* strains, although D92S and D56S differ only in eight amino acid residues in the juxtamembrane region. These results suggest that the LRR domain is an important determinant of XA3/XA26-mediated race-specific resistance, but the juxtamembrane region and kinase domain also influence the recognition of XA3/XA26 in disease resistance.

Activities of development-controlled *R* genes are associated with their expression levels

To analyze the developmentally controlled activity of *Xa3/Xa26* and *Xa21*, the *Xoo* resistance of rice plants carrying *Xa3/Xa26* or *Xa21* at different growth stages was examined at the same time (Fig. 4). IRBB21 plants carrying *Xa21* were susceptible or moderately susceptible to *Xoo* strains at the seedling stage (two- and four-leaf stages), but showed gradually increased resistance to the incompatible *Xoo* strains PXO61 and PXO99 as the plants grew, and this resistance reached a plateau at the maximum-tillering stage. Although susceptible to the compatible *Xoo* strain PXO341, IRBB21 showed a similar response to PXO341 infection as to PXO61 or PXO99 infection in different growth stages; IRBB21 was more susceptible to PXO341 in seedling stage than in adult stage (Fig. 4). The response of rice lines Minghui 63 and IRBB3 carrying *Xa3/Xa26* to the incompatible strains PXO61 and PXO341 and compatible strain PXO99 was similar to IRBB21 (Fig. 4). However, the transgenic lines Rb49 and D49OM6 carrying *Xa3/Xa26* and *Xa21*, respectively, showed stable resistance to incompatible strains from the two-leaf stage to the heading stage. These results suggest that Mudanjiang 8 background may facilitate the function of *Xa3/Xa26* and *Xa21*.

Our previous study suggested that the influence of developmental stage on *Xa3/Xa26* activity is associated with the expression of this gene, and increased expression enhances *Xa3/Xa26*-mediated resistance (Cao et al. 2007a). The responses of Minghui 63 to incompatible strains PXO61 and PXO341 observed in this study further confirmed our previous findings. The gradually reduced lesion areas with the growth of Minghui 63 after PXO61 or PXO341 infection (Fig. 4) fit perfectly with the gradually increased expression of *Xa3/Xa26* (Cao et al. 2007a).

To determine whether developmentally controlled *Xa21* activity was also associated with the expression of this gene, we examined *Xa21* expression at different developmental stages. Rice line IRBB21 and transgenic line D49OM6 carrying *Xa21* were grown with staggered planting so that RNA samples were simultaneously obtained from different lines at different growth stages (Fig. 5). *Xa21* belongs to a multigene family (Song et al. 1995). We

Table 1 Resistance spectra (percent lesion area) at the booting stage of transgenic plants and varieties carrying *Xa3/Xa26*, *Xa21*, or chimeric genes

Rice line[a]	*Xoo* strain							
	PXO61	PXO79	PXO71	PXO99	PXO280	PXO341	JL691	KS-1-21
IR24	53.0 ± 5.9	52.3 ± 4.4	53.1 ± 4.2	48.1 ± 11.0	42.5 ± 8.8	44.4 ± 3.8	55.5 ± 7.2	57.3 ± 8.0
IRBB21 (*Xa21*)	6.9 ± 6.4	3.9 ± 1.5	3.6 ± 2.1	8.4 ± 3.6	6.0 ± 6.8	26.3 ± 3.8	7.0 ± 6.8	15.2 ± 6.3
IRBB3 (*Xa3/Xa26*)	4.9 ± 1.6	5.3 ± 1.8	4.9 ± 1.2	37.1 ± 5.8	5.0 ± 2.3	7.0 ± 1.3	4.4 ± 0.8	36.3 ± 2.8
Minghui 63 (*Xa3/Xa26*)	30.0 ± 9.0	17.3 ± 2.6	15.5 ± 5.8	49.3 ± 8.7	24.0 ± 5.1	24.3 ± 4.4	4.9 ± 0.7	52.1 ± 6.6
Mudanjiang 8	62.9 ± 6.4	78.5 ± 9.0	65.3 ± 8.5	71.6 ± 12.5	70.5 ± 17.3	58.5 ± 14.3	62.0 ± 6.1	92.7 ± 5.8
Rb49 (*Xa3/Xa26*)	3.9 ± 2.8*	3.4 ± 1.4*	2.5 ± 1.1*	50.0 ± 6.5	52.7 ± 15.5	10.1 ± 8.3*	4.2 ± 1.5*	94.6 ± 4.9
D49OM6 (*Xa21*)	4.1 ± 1.5*	8.0 ± 3.4*	3.1 ± 1.6*	4.8 ± 1.6*	2.1 ± 1.4*	31.1 ± 4.8*	3.8 ± 1.4*	9.7 ± 7.4*
D52SM20 (*D52S*)	76.6 ± 8.1	82.3 ± 12.7	70.6 ± 10.9	58.7 ± 23.6	53.9 ± 24.1	58.2 ± 17.5	63.6 ± 11.7	90.0 ± 7.4
D56SM6 (*D56S*)	4.7 ± 2.3*	9.2 ± 2.1*	26.4 ± 5.6*	30.0 ± 6.4*	50.5 ± 13.3	4.4 ± 2.5*	7.2 ± 3.3*	60.7 ± 10.3*
D57SM5 (*D57S*)	9.2 ± 2.0*	14.0 ± 2.7*	13.9 ± 3.9*	8.2 ± 3.9*	15.0 ± 3.6*	46.5 ± 17.4	8.1 ± 3.0*	24.9 ± 6.5*
D92SM1 (*D92S*)	71.7 ± 3.6	78.1 ± 9.1	71.9 ± 11.5	68.2 ± 7.4	72.0 ± 9.3	66.8 ± 8.3	65.3 ± 6.7	88.9 ± 3.1

* Significant difference ($P < 0.01$) was detected compared with wild-type Mudanjiang 8

[a] Rb49, D49OM6, D52SM20, D56SM6, D57SM5, and D92SM1 are homozygous transgenic lines carrying a single copy of transgene in the genetic background of susceptible *japonica* variety Mudanjiang 8. IRBB21 and IRBB3 are near-isogenic lines with the susceptible *indica* variety IR24 as the recurrent parent. Minghui 63 is an *indica* variety

designed primers that recognized only the kinase domain of *Xa21* in both IRBB21 and Mudanjiang 8 backgrounds for qRT-PCR analysis. *Xa21* expression was very low at the two-leaf stage, gradually increased with development, and reached the highest level at the maximum-tillering stage in IRBB21. The expression pattern of *Xa21* was consistent with gradually increased resistance accompanying development in IRBB21 (Fig. 4). The *Xa21* transcript levels in D49OM6 were approximately 7.6-, 5.5-, 2.8-, and 2.5-fold higher than those in IRBB21 at two-leaf, four-leaf, maximum-tillering, and booting stages, respectively. This high expression level of *Xa21* was consistent with the stable resistance of D49OM6 throughout development (Fig. 4). These results suggest that development-regulated *Xa21* activity is also associated with its expression level.

Discussion

Development-controlled disease resistance has long been observed in many plant–pathogen systems (e.g., Bateman and Lumsden 1965; Griffey and Leach 1965). Different mechanisms of development-regulated resistance have been reported. Calcium content and the nature of the pectic substances were associated with age-related susceptibility to *Rhizoctonia solani* in bean (Bateman and Lumsden 1965). Accumulation of secondary metabolites, such as phytoalexins, has been reported in development-regulated resistance in cotton and pepper (Hunter et al. 1978; Hwang 1995). Development-regulated resistance was also observed in Arabidopsis, which becomes more resistant to virulent *Pseudomonas syringe* as plants develop (Kus et al. 2002); this response is independent of induced systemic resistance and systemic acquired resistance, but it is dependent on the accumulation of salicylic acid (SA). Studies also indicated that age-related resistance is controlled by single resistance genes in cereal, cowpea, maize, and Arabidopsis (Roelfs 1984; Heath 1994; Abedon and Tracy 1996; Panter et al. 2002).

However, little is known about the biochemical and molecular mechanisms involved in this regulation. *Cf-9B*, a paralog of *Cf-9* from tomato, only confers adult disease resistance. Promoter swapping analysis showed that the transcriptional regulation of *Cf-9B* did not account for the late onset of *Cf-9B*-mediated resistance (Panter et al. 2002). Our previous study showed that development-regulated *Xa3/Xa26*-mediated resistance to *Xoo* is dosage-dependent, which in turn influences the expression of *NH1* and *OsWRKY13*, two important regulators functioning actively in two SA-dependent defense signaling pathways (Cao et al. 2007a). The present results further confirm that the gradually enhanced resistance of rice to *Xoo* is related to the gradually increased expression of *Xa3/Xa26* during development (Cao et al. 2007a). In addition, our results suggest that *Xa21*-mediated developmentally controlled *Xoo* resistance is related to its expression level. Increasing *Xa21* expression can erase the influence of development on the function of *Xa21* at the seedling stage. However, Century et al. (1999) reported that *Xa21* expression was independent of rice developmental stage. The inconsistency of our results with that reported by Century et al. (1999) may be due to different techniques used for analyzing *Xa21*

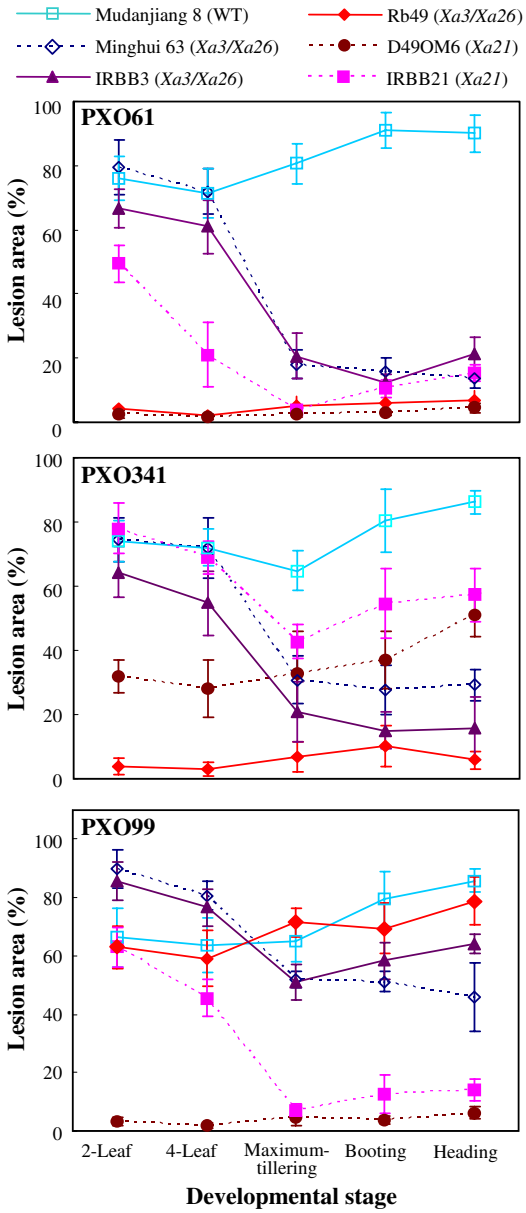


Fig. 4 Developmental stages influence the function of *R* genes. Rb49 and D49OM6 are transgenic lines with the genetic background of susceptible Mudanjiang 8. Minghui 63, IRBB3, and IRBB21 are original lines. IRBB3 and IRBB21 are near-isogenic lines. Each point represents mean (5–20 replicates) ± standard deviation

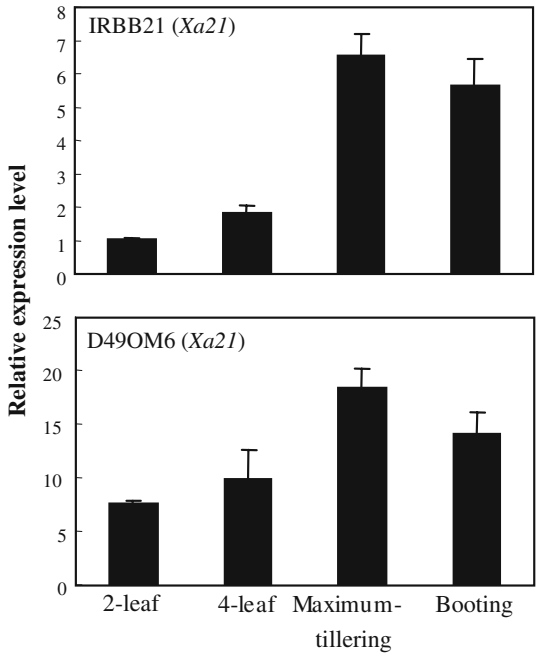


Fig. 5 Developmental stage influences *Xa21* expression. Bars represent mean (three replicates) ± standard deviation. The expression level of *Xa21* in each sample is relative to that in IRBB21 at two-leaf stage

expression. The real time qRT-PCR performed in our study should be more sensitive and accurate than RT-PCR applied in the previous study. In addition, the plants used for analyzing *Xa21* expression were grown with staggered planting so that RNA samples were obtained from plants in different developmental stages at the same time in our study, which reduced the influence of environmental factors that might affect *Xa21* expression. Furthermore, other observations also support that *Xa21*-mediated resistance shows a gene dosage effect. Transgenic lines carrying *Xa21* showed a greater level of resistance than the *Xa21* gene donor line, which was suggested to be due to multiple copies of the *Xa21* transgene (Zhang et al. 1998). The protein level of XA21 appears to contribute to the resistance (Xu et al. 2006). However, XA21 protein level was developmentally regulated; this protein was more stable at the seedling stage, in which XA21 did not confer resistance. Thus, Xu et al. (2006) explained the development-controlled resistance conferred by *Xa21* using the hypothesis that developmental regulation of *Xa21*-mediated resistance is controlled by a downstream factor(s) (Century et al. 1999). These results suggest that more than one mechanism may be involved in the developmental regulation of *Xa21*-mediated resistance. Although taken together these data suggest that development-controlled disease resistance is regulated by various mechanisms, it is worth examining whether differential expression of *R* genes during development is a general mechanism in *R*-gene-mediated development-controlled resistance.

In general, as a beta-sheet/beta-turn structure, LRR motifs can recognize and interact directly or indirectly with pathogen-associated molecular patterns (PAMPs) or effectors delivered by pathogens and transmit defense signaling through conformational change. Accumulating evidence suggests that the LRR domains of nucleotide-binding site-LRR type and LRR-TM type R proteins play a critical role in determining resistance specificity (Ellis and Jones 1998; DeYoung and Innes 2006). However, FLS2 and ERF, two

LRR receptor kinase proteins in Arabidopsis, provide non-specific resistance by recognizing conserved bacterial PAMPs fls22 and erf18 (Gomez-Gomez and Boller 2000; Zipfel et al. 2006). Thus far, XA3/XA26 and XA21 are the only two characterized LRR receptor kinase-type proteins that confer race-specific resistance. The LRR domains of XA3/XA26 and XA21 are subject to positive selection; in addition, the LRR domains of the XA3/XA26 family appear to have been subject to a higher rate of mutation (Sun et al. 2006). Point mutations and positive selection in LRR were largely responsible for the diversity of adaptations for variation of pathogens. The co-evolution of the LRR domains of XA3/XA26 and XA21 with the variation of pathogens suggests that they must be involved in race-specific recognition. This hypothesis is supported by the observation that *Xa21D*, a member of *Xa21* family that does not express the kinase domain, mediates partial and race-specific resistance to *Xoo* (Wang et al. 1998). The present results further support this hypothesis. Domain swapping indicates that the LRR domains of the XA3/XA26 and XA21 are important determinants of race-specific recognition.

Although increasing evidence suggests that the juxtamembrane region plays an essential role for the functions of LRR receptor kinase proteins, there is limited information about its role in plant disease resistance. Three autophosphorylated residues, Ser686, Thr688, and Ser689, within the cytoplasmic juxtamembrane region were found to be related with the stability of XA21. Rice plants carrying mutated at these three residues were compromised in their response to an avirulent *Xoo* race compared to a resistant control, but showed enhanced resistance compared to susceptible wild type (Xu et al. 2006). Our results suggest that in addition to Ser686, Thr688, and Ser689, other residues of the cytoplasmic juxtamembrane region may also influence the function of XA21. D52S and D57S differ only in 14 amino acid residues, including Thr688 and Ser689. Plants carrying D52S completely lost the ability to recognize avirulent *Xoo* races compared to resistant plants carrying D57S and susceptible wild type.

The juxtamembrane region may influence the function of XA3/XA26 and XA21 in two ways in addition to influencing the protein stability of XA21 (Xu et al. 2006). First, the juxtamembrane region may influence pathogen recognition specificity. Plants carrying *Xa3/Xa26* were compatible (susceptible) to *Xoo* strains PXO99, PXO280, and KS-1-21 and plants carrying *Xa21* were compatible to *Xoo* strain PXO341 and incompatible (resistant) to PXO99, PXO280, and KS-1-21. Plants carrying chimeric protein D56S, which contains a partial cytoplasmic juxtamembrane region of XA21, showed enhanced resistance to PXO99, PXO280, and KS-1-21 compared to plants carrying XA3/XA26 in the same genetic background. However, plants carrying chimeric D57S, which contains an intact juxtamembrane region of XA21, were still susceptible to PXO341. Second, the cytoplasmic juxtamembrane region may be associated with the function of kinase domains in XA3/XA26 and XA21. This hypothesis is supported by evidence that D92S, with an intact juxtamembrane region of XA3/XA26 and kinase domain of XA21, could not mediate resistance, but D56S, which differs from D92S in only eight amino acids in the juxtamembrane region, conferred *Xoo* resistance. Thus, the race-specific recognition conferred by XA3/XA26 and XA21 appears to be regulated by at least the LRR domain and juxtamembrane region.

Acknowledgments We thank Dr. Pamela Ronald of the University of California at Davis for providing pC822 clone and the Center for the Application of Molecular Biology to International Agriculture for providing vector pCAMBIA1301. This work was supported by grants from the National Program on the Development of Basic Research in China, the National Program of High Technology Development of China, and the National Natural Science Foundation of China.

References

Abedon BG, Tracy WF (1996) Corngrass1 of maize (*Zea mays* L) delays development of adult plant resistance to common rust (*Puccinia sorghi* Schw) and European corn borer (*Ostrinia nubilalis* hubner). J Hered 87:219–223

Bateman DF, Lumsden RD (1965) Relation of calcium content and nature of pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology 55:734–738

Cao Y, Ding X, Cai M, Zhao J, Lin Y, Li X, Xu C, Wang S (2007a) Expression pattern of a rice disease resistance gene *Xa3/Xa26* is differentially regulated by the genetic backgrounds and developmental stages that influence its function. Genetics 177:523–533

Cao Y, Duan L, Li H, Sun X, Zhao Y, Xu C, Li X, Wang S (2007b) Functional analysis of *Xa3/Xa26* family members in rice resistance to *Xanthomonas oryzae* pv. *oryzae*. Theor Appl Genet 115:887–895

Century KS, Lagman RA, Adkisson M, Morlan J, Tobias R, Schwartz K, Smith A, Love J, Ronald PC, Whalen MC (1999) Developmental control of *Xa21*-mediated disease resistance in rice. Plant J 20:231–236

Chen H, Wang S, Zhang Q (2002) New gene for bacterial blight resistance in rice located on chromosome 12 identified from Minghui 63, an elite restorer line. Phytopathology 92:750–754

Chu Z, Ouyang Y, Zhang J, Yang H, Wang S (2004) Genome-wide analysis of defense-responsive genes in bacterial blight resistance of rice mediated by the recessive *R* gene *xa13*. Mol Genet Genom 271:111–120

Chu Z, Yuan M, Yao L, Ge X, Yuan B, Xu C, Li X, Fu B, Li Z, Bennetzen J, Zhang Q, Wang S (2006) Promoter mutations of an essential gene for pollen development result in disease resistance in rice. Gene Dev 20:1250–1255

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defense responses to infection. Nature 411:826–833

DeYoung BJ, Innes RW (2006) Plant NBS-LRR proteins in pathogen sensing and host defense. Nat Immunol 7:1243–1249

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S (2008) Activation of the indole-3-acetic acid-amido synthetase GH3–8 suppresses expansin expression and promotes salicylate- and

jasmonate-independent basal immunity in rice. Plant Cell 20:228–240

Ellis J, Jones D (1998) Structure and function of proteins controlling strain-specific pathogen resistance in plants. Curr Opin Plant Biol 1:288–293

Ellis JG, Lawrence GJ, Luck JE, Dodds PN (1999) Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11:495–506

Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5:1003–1011

Griffey RT, Leach JG (1965) Influence of age of tissue on development of bean anthracnose lesions. Phytopathology 55:915–918

Gu KY, Yang B, Tian DS, Wu LF, Wang DJ, Sreekala C, Yang F, Chu ZQ, Wang GL, White FF, Yin ZC (2005) *R* gene expression induced by a type-III effector triggers disease resistance in rice. Nature 435:1122–1125

Heath MC (1994) Genetics and cytology of age-related resistance in North American cultivars of cowpea (*Vigna unguiculata*) to the cowpea rust fungus (*Uromyces vignae*). Can J Bot 72:575–581

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes—gene-splicing by overlap extension. Gene 77:61–68

Hu K, Qiu D, Shen X, Li X, Wang S (2008) Isolation and manipulation of quantitative trait loci for disease resistance in rice using a candidate gene approach. Mol Plant 1:786–793

Hunter RE, Halloin JM, Veech JA, Carter WW (1978) Terpenoid accumulation in hypocotyls of cotton seedlings during aging and after infection by *Rhizoctonia solani*. Phytopathology 68:347–350

Hwang BK (1995) Effects of age-related resistance and metalazyl on capsidiol production in pepper plants infected with *Phytophthora capsici*. In: Daniel M, Purkayastha RP (eds) Handbook of phytoalexin methobolism and action. Marcel Dekker, New York, pp 503–523

Iyer AS, McCouch SR (2004) The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance. Mol Plant Microbe Interact 17:1348–1354

Kus JV, Zaton K, Sarkar R, Cameron RK (2002) Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell 14:479–490

Lin YJ, Zhang QF (2005) Optimising the tissue culture conditions for high efficiency transformation of *indica* rice. Plant Cell Rep 23:540–547

Narayanan NN, Baisakh CM, Vera Cruz CM, Gnanamanickam SS, Datta K, Datta SK (2002) Molecular breeding for the development of blast and bacterial blight resistance in rice cv. IR50. Crop Sci 42:2072–2079

Ogawa TR, Tabien E, Busto GA, Khush GS, Mew TW (1986) The relationships between *Xa-3, Xa-4, and Xa-4^b* for resistances to rice bacterial blight. Rice Genet Newsl 3:83–84

Panter SN, Hammond-Kosack KE, Harrison K, Jones JDG, Jones DA (2002) Developmental control of promoter activity is not responsible for mature onset of *Cf-9B*-mediated resistance to leaf mold in tomato. Mol Plant-Microbe Interact 15:1099–1107

Qiu D, Xiao J, Ding X, Xiong M, Cai M, Cao CL, Li X, Xu C, Wang S (2007) *OsWRKY13* mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. Mol Plant Microbe Interact 20:492–499

Roelfs AP (1984) Race specificity and methods of study. In: Bushnell WR, Roelfs AP (eds) The cereal rusts, vol 1: origins, specificity, structure and physiology. Academic Press, San Diego, pp 131–164

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804–1806

Sun X, Cao Y, Yang Z, Xu C, Li X, Wang S, Zhang Q (2004) *Xa26*, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein. Plant J 37:517–527

Sun X, Cao Y, Wang S (2006) Point mutations with positive selection were a major force during the evolution of a receptor-kinase resistance gene family of rice. Plant Physiol 140:998–1008

Van der Hoorn RAL, Roth R, De Wit PJG (2001) Identification of distinct specificity determinants in resistance protein Cf-4 allows construction of a Cf-9 mutant that confers recognition of avirulence protein AVR4. Plant Cell 13:273–285

Wang GL, Ruan DL, Song WY, Sideris S, Chen L, Pi LY, Zhang S, Zhang Z, Fauquet C, Gaut BS, Whalen MC, Ronald PC (1998) Xa21D encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. Plant Cell 10:765–779

Wang G, Ding X, Yuan M, Qiu D, Li X, Xu C, Wang S (2006) Dual function of rice *OsDR8* gene in disease resistance and thiamine accumulation. Plant Mol Biol 60:437–449

Xiang Y, Cao Y, Xu C, Li X, Wang S (2006) *Xa3*, conferring resistance for rice bacterial blight and encoding a receptor kinase-like protein, is the same as *Xa26*. Theor Appl Genet 113:1347–1355

Xu WH, Wang YS, Liu GZ, Chen XH, Tinjuangjun P, Pi LY, Song WY (2006) The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. Plant J 45:740–751

Yoshimura S, Yamanouchi U, Katayose Y, Toki S, Wang ZX, Kono I, Kurata N, Yano M, Iwata N, Sasaki T (1998) Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. Proc Natl Acad Sci USA 95:1663–1668

Yuan B, Shen X, Li X, Xu C, Wang S (2007) Mitogen-activated protein kinase OsMPK6 negatively regulates rice disease resistance to bacterial pathogens. Planta 226:953–960

Zhang S, Song WY, Chen L, Ruan D, Taylor N, Ronald P, Beachy R, Fauquet C (1998) Transgenic elite *Indica* rice varieties, resistant to *Xanthomonas oryzae* pv. *oryzae*. Mol Breed 4:551–558

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125:749–760