Theor Appl Genet (2006) 112: 455–461
DOI 10.1007/s00122-005-0145-6

ORIGINAL PAPER

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Targeting *xa13*, a recessive gene for bacterial blight resistance in rice

Received: 3 August 2005 / Accepted: 23 October 2005 / Published online: 17 November 2005

Abstract Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most serious diseases of rice worldwide. Thirty bacterial blight resistance (*R*) genes (21 dominant genes and 9 recessive genes) in rice have been identified. They are the main sources for the genetic improvement of rice for resistance to *Xoo*. However, little is known about the recessive *R* genes. To clone and characterize the recessive *R* genes, we fine-mapped *xa13*, a fully recessive gene for *Xoo* resistance, to a DNA fragment of 14.8 kb using the map-based cloning strategy and a series of sequence-based molecular markers. Sequence analysis of this fragment indicated that this region contains only two apparently intact candidate genes (an extensin-like gene and a homologue of nodulin MtN3) and the 5′ end of a predicted hypothetical gene. These results will greatly facilitate the isolation and characterization of *xa13*. Four PCR-based markers, E6a, SR6, ST9 and SR11 that were tightly linked to the *xa13* locus, were also developed. These markers will be useful tools for the marker-assisted selection of *xa13* in breeding programs.

Communicated by D. J. Mackill

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Introduction

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases of rice worldwide. This disease is not only widespread throughout Asia but also occurs in Australia, the United States and in several rice growing countries of Latin America and Africa. Yield losses caused by *Xoo* typically range from 20 to 30% and can be as high as 50% in some areas of Asia (Ou 1985; Adhikari et al. 1995). The use of resistant cultivars is the most economical and effective way to control this disease. Currently, 30 major genes conferring host resistance against various strains of *Xoo* have been identified and designated in a series from *Xa1* to *Xa29*. These include 21 dominant *R* genes and 9 recessive *R* genes, though there has been some confusion in the designation of these loci (Sidhu and Khush 1978; Ogawa et al. 1986a, b, c, 1988; Lin et al. 1996; Nagato and Yoshimura 1998; Zhang et al. 1998; Khush and Angeles 1999; Gao et al. 2001; Chen et al. 2002; Lee et al. 2003; Yang et al. 2003; Tan et al. 2004).

To date, more than 40 plant *R* genes have been cloned and characterized, including five *R* genes for rice bacterial blight resistance, *Xa1*, *xa5*, *Xa21*, *Xa26* and *Xa27* (Song et al. 1995; Yoshimura et al. 1998, Iyer and McCouch 2004; Sun et al. 2004; Gu et al. 2005). Only three of these 40 cloned genes are recessive (Buschges et al. 1997; Martin et al. 2003; Iyer and McCouch 2004). The products of the most dominant *R* genes appear to function as receptors that interact directly or indirectly with pathogen elicitors to initiate hypersensitive responses (Martin et al. 2003). However, the few recessive *R* genes that have been cloned each have very different structures, suggesting that they function differently.

The products of the three characterized recessive *R* genes, *mlo*, *RRS1-R* and *xa5*, are all quite different. The dominant *Mlo* allele encodes a transmembrane protein that acts as a negative regulator of the defense response of barley to its fungal pathogen, *Erysiphe graminis* f. sp. Hordei, and the recessive allele, *mlo*, is a loss-of-function mutant (Kim et al. 2002). The *RRS1-R* gene from *Arabidopsis* confers resistance to *Ralstonia solanacearum* and encodes a novel nucleotide binding-leucine rich repeat-WRKY protein, which is presumed to function in the nucleus (Deslandes et al. 2002; Lahaye 2002). However, *RRS1-R* behaves as a dominant *R* gene in transgenic plants. The rice *xa5* gene confers resistance to the Philippine *Xoo* races 1, 2, 3 and 5 (Li et al. 2001) and encodes a gamma subunit of transcription factor IIA (Iyer and McCouch 2004). The *xa5* gene is actually partially dominant, as the F_1 plants heterozygous at the *xa5* locus have lesion lengths intermediate between its resistant and susceptible near-isogenic parents (Li et al. 2001).

The *xa13* gene is fully recessive, conferring resistance only in the homozygous status (Khush and Angeles 1999). This gene specifically confers resistance to the Philippine *Xoo* race 6; the most virulent race and one not overcome by most reported *R* genes. The *xa13* gene was first discovered in the rice variety BJ1 and mapped on the long arm of rice chromosome 8 (Ogawa et al.1987; Zhang et al. 1996; Sanchez et al.1999). It interacts strongly with other *R* genes such as *xa5*, *Xa4* and *Xa21* (Li et al. 2001). Small-scale gene expression studies and pathogen-induced subtractive cDNA library analysis have revealed that some defense-responsive genes activated in *xa13*-mediated resistance are not involved in resistance that is mediated by dominant *R* genes (*Xa4*, *Xa10* and *Xa26*) (Zhou et al. 2002; Wen et al. 2003; Chu et al. 2004). These results suggest that *xa13* functions differently from other *R* genes in initiating resistance against *Xoo*. Thus, fine mapping and molecular cloning of the *xa13* gene would significantly enhance our understanding of disease resistance pathways in rice.

We report here the fine mapping of *xa13* to a 14.8-kb DNA fragment. Sequence analysis of this fragment indicated that this region contained two complete open reading frames and the 5′ end of a predicted hypothetical gene. These results set the foundation for the isolation and characterization of this gene. In addition, four PCR-based molecular markers, all tightly flanking the *xa13* locus, were developed for the direct marker-assisted selection of *xa13* in breeding programs.

Material and methods

Materials

Three F_2 populations from the cross between a susceptible rice line, IR24 (*Oryza sativa* ssp. *indica*), and its near-isogenic line, IRBB13, carrying the *xa13* gene were used for the fine mapping of *xa13*. The first population consisted of 250 individuals highly resistant (lesion length < 3 cm) to Philippine *Xoo* race 6, the second consisted of 6,000 random individuals, and the third comprised 1,972 random individuals. The first population was originally constructed to map *xa13* using the *R*-gene homologue strategy. However, mapping results showed that the *R*-gene homologue did not co-segregate with *xa13*. Thus, the second population was constructed for the fine mapping of *xa13* based on the marker information of physical mapping *xa13* (Sanchez et al. 1999). The third population was supplemented to analyze the recombinant events that occurred on one side of the *xa13* locus after having noticed previous mis-mapping information on this side. A bacterial artificial chromosome (BAC) library constructed with genomic DNA from the rice cultivar Minghui 63 (*O. sativa* ssp. *indica*), with an average insert length of 150 kb (Peng et al. 1998), was used for the construction of the physical map covering the *xa13* region.

Disease evaluation

At the booting stage, five of the uppermost fully expanded leaves of each plant were inoculated with Philippine *Xoo* race 6 (PXO99) by the leaf-clipping method (Kauffman et al. 1973). The bacterial inoculum was prepared as described previously (Lin et al. 1996). Reactions of individual F_2 plants to the pathogen were evaluated 14–21 days after inoculation by measuring the lesion length (in cm).

DNA sequencing and sequence analysis

A shotgun approach was used to determine the nucleotide sequence of BAC clones. The M13 universal forward and reverse primers and the BigDye Terminator Cycle Sequencing v2.0 kit (Applied Biosystems, Foster City, CA, USA) were used for sequencing. Sequences were assembled using the computer program Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, USA). DNA sequence similarity analysis was performed using BLAST programs, including BLASTN and BLASTX (Altschul et al.1997). Candidate genes were predicted by the combined use of FGENESH (http://www.softberry.com), GeneMark.hmm (http://dixie.biology.gatech.edu/Genemark/eukhmm.cgi) and GENSCAN programs (http://genes.mit.edu/GENSCAN.html, Burge and Karlin 1997).

Genotyping and physical mapping

The three F_2 populations were genotyped with four restriction fragment length polymorphism (RFLP) markers: RG136 and S14003, based on previous map-

ping studies (Zhang et al. 1996; Sanchez et al. 1999), and RP1 and RP2, developed from BAC clones 22E06 and 23P23. The cleaved amplification polymorphism sequence (CAPS) marker E6a from BAC clone 22E06 and the simple sequence repeat (SSR) marker SR11 from BAC clone 44F01 were also developed and used to genotype the three F_2 populations. The PCR primers and restriction enzyme were E6aF (5′-agctcaagagcatctccgtc-3′), E6aR (5′-gtctgtgaaggaactttctcgc-3′) and *Hpa*II for marker E6a. A 580-bp fragment was amplified from both IR24 and IRBB13 using E6aF and E6aR. After digestion of the 580-bp fragment using *Hpa*II, two fragments (106 and 474 bp) from IR24 and three fragments (106, 192 and 282 bp) from IRBB13 were observed by electrophoresis on 2% agarose gel. The PCR primers for marker SR11 were SR11F (5′-tgtcctttgccttcttcctc-3′) and SR11R (5′-ccggatggatctcctgtcta-3′), which amplified a 272-bp fragment from IR24 and a larger fragment from IRBB13. The PCR reaction mixture for the PCR-based markers contained 10–20 ng template DNA, 50 ng of each primer, 0.1 mM dNTPs, 1× buffer (10 mM Tris, pH 9.0, 50 mM KCl, and 1% Triton X-100), 1.8 mM $MgCl_2$, and 1 U Taq DNA polymerase in a final volume of 20 μl. The DNA was amplified at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min for a total of 35 cycles.

Markers E6a and S14003 were used as initial probes to screen the BAC filters prepared as described previously (Liu et al. 2001) for construction of the physical map covering the *xa13* region. The subclones from the positive BAC clones, in turn, were used as probes for chromosome walking. The overlapping relationship of the BAC clones was fingerprinted with restriction enzyme *Hin*dIII. After three steps from both the E6a and S14003 sides, the same BAC clone was identified, indicating that the contig covering the *xa13* region was complete.

For fine physical mapping of *xa13*, a series of markers was further developed from the BAC clones covering the *xa13* region. These included three RFLP markers of shotgun clones (RP3, RP4 and RP5) from BAC clone 21H14, SSR marker SR6 from BAC clone 21H14, three RFLP markers of shotgun clones (RP7, RP8 and RP10) from BAC clone 14L03, and CAPS marker ST9 from BAC clone 14L03. The PCR primers for SR6 were SR6F (5′-acagatccagctccagcttc-3′) and SR6R (5′-cgttgacgaggagtttggtt-3′), which amplified a 249-bp fragment from IR24 and a smaller fragment from IRBB13. The PCR primers and restriction enzyme for ST9 were ST9F (5′-cattggatgggttgacacag-3′), ST9R (5′-tagcttcgcgtcttggagat-3′) and *Xho*I. A 700-bp fragment was amplified from both IR24 and IRBB13 using ST9F and ST9R. After digestion of the 700-bp amplicons using *Xho*I, two fragments (106 and 594 bp) from IR24 and one fragment (700 bp, no digestion) from IRBB13 were observed by electrophoresis on an agarose gel.

Results and discussion

Fine and physical mapping of *xa13*

The *xa13* locus was reportedly located on the long arm of rice chromosome 8 (Zhang et al. 1996; Sanchez et al. 1999). Using the first F_2 population, we confirmed the location of *xa13* between the RFLP markers RG136 and S14003 (Fig. 1). A CAPS marker, E6a, was mapped between RG136 and the *xa13* locus, putting the *xa13* locus in a region of 1.6 and 0.4 cM between E6a and S14003, respectively (Fig. 1).

A physical map that covers the region flanked by markers E6a and S14003 was constructed by chromosome walking using Minghui 63 BAC clones. This map was composed of five non-redundant and overlapping BAC clones (22E06, 23P23, 30A21, 39N05 and 44F01) (Fig. 1). Two RFLP markers, RP1 and RP2, developed from BAC clones 22E06 and 23P23, respectively, were mapped to one side of the *xa13* locus. One BAC clone, 44F01, had a similar DNA fingerprint pattern to two other BAC clones, 21H14 and 14L03, from the IR64 BAC library, confirming the physical location of *xa13* in the BAC clones (Sanchez et al. 1999; Fig. 1 and Fig. 2). Because both Minghui 63 and IR64 are susceptible to the *Xoo* strain PXO99, the three BAC clones are expected to carry the dominant allele of *xa13* for susceptibility to PXO99. We then sequenced BAC clone 14L03. It had an insert size of 66.6 kb. BAC clones 21H14 and

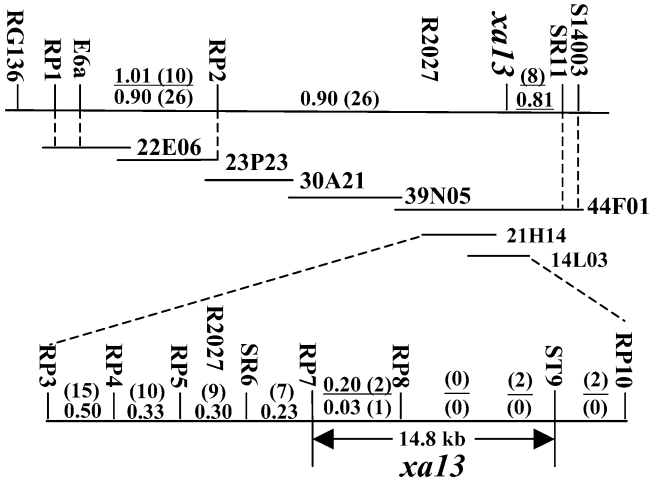

Fig. 1 A contig map covering the *xa13* region. The *long horizontal lines* indicate the region containing the *xa13* locus. The *short horizontal lines* represent the BAC clones. The numbers between molecular markers indicate the genetic distances in centiMorgan and the number of recombination events (in *parentheses*) detected between the *xa13* locus and the corresponding markers. The *underlined numbers* represent genetic distances and recombination events detected in the third F_2 population and *other numbers* represent genetic distances and recombination events detected in the second F_2 population. The *dashed vertical lines* between the markers and BAC clones indicate that hybridization between markers and BAC clones was verified

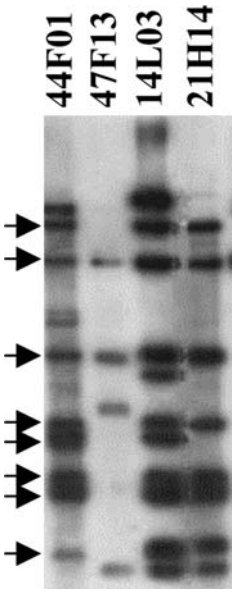

Fig. 2 Southern-blot analysis of the BAC clones covering the *xa13* region. BAC clone 44F01 and 47F13 were from rice cultivar Minghui 63 and BAC clones 14L03 and 21H14 were from rice cultivar IR64. These BAC clones were fingerprinted with *Hin*dIII. *Arrows* indicate that 44F01 has a similar restriction fragment pattern to 14L03 and/or 21H14

44F01 had insert sizes of approximately 90 and 140 kb, respectively. About 79 kb of 21H14 and 21 kb of 44F01 were sequenced.

Targeting of *xa13* to a single DNA fragment

Nine markers, RP3, RP4, RP5, SR6, RP7, RP8, ST9, RP10 and SR11 (Fig. 1), were developed from the three sequenced BAC clones to fine-map *xa13* using the second and third F_2 populations. Twenty-six F_2 recombinants, showing recombination events between the *xa13* locus and marker E6a from the second F_2 population, were first screened using eight of the nine markers. Markers RP3, RP4, RP5, SR6 and RP7 detected 15, 10, 9, 7 and 1 recombination events, respectively, while RP8, ST9 and RP10 co-segregated completely with *xa13* in the 26 F_2 plants, placing *xa13* 0.03 cM from RP7 (Table 1).

Four markers, RP7, RP8, ST9 and RP10, were further used to screen the recombinant F_2 individuals detected by E6a and SR11 in the third population (Table 2). Recombination was detected between RP7 and *xa13* in two of the ten plants identified by E6a; but markers RP8, ST9 and RP10 co-segregated with *xa13* in the ten plants identified using marker E6a. Two of the eight recombinant individuals identified by SR11

Table 1 Marker genotypes and resistance phenotypes (reactions to the *Xoo* strain PXO99) of 26 recombinant individuals from the second F_2 population ($n = 6{,}000$)

Individuals	Phenotype	Marker genotype									
		E6a	RP2	RP3	RP4	RP5	SR6	RP7	RP8	ST9	RP10
4E12	R	**H**	**H**	R	R	R	R	R	R	R	R
08C01	S	**R**	**R**	H	H	H	H	H	H	H	H
13G06	R	**H**	**H**	R	R	R	R	R	R	R	R
17F09	R	**H**	**H**	R	R	R	R	R	R	R	R
21H07	S	**R**	**R**	H	H	H	H	H	H	H	H
25H05	S	**R**	**R**	H	H	H	H	H	H	H	H
26A12	R	**H**	**H**	R	R	R	R	R	R	R	R
28R06	R	**H**	**H**	R	R	R	R	R	R	R	R
43R01	R	**H**	**H**	R	R	R	R	R	R	R	R
51R05	R	**H**	**H**	R	R	R	R	R	R	R	R
53F01	R	**H**	**H**	R	R	R	R	R	R	R	R
3H06	S	**R**	**R**	**R**	H	H	H	H	H	H	H
5D01	R	**H**	**H**	**H**	R	R	R	R	R	R	R
9E04	R	**H**	**H**	**H**	R	R	R	R	R	R	R
10H12	R	**H**	**H**	**H**	R	R	R	R	R	R	R
23H01	R	**H**	**H**	**H**	R	R	R	R	R	R	R
14F01	R	**H**	**H**	**H**	**H**	R	R	R	R	R	R
31F04	R	**H**	**H**	**H**	**H**	**H**	R	R	R	R	R
57H11	R	**H**	**H**	**H**	**H**	**H**	R	R	R	R	R
1A10	R	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
7E01	R	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
16H02	R	**S**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
32H12	R	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
35E08	R	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
39A12	R	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R	R
61C05	R	**H**	**H**	**H**	**H**	**H**	**H**	**H**	R	R	R
Total number of recombination events		26	26	15	10	9	7	1	0	0	0

R, S and H stand for the homozygous resistant, homozygous susceptible and heterozygote genotypes, respectively, of the F_2 recombinants from the cross between the resistant and susceptible near-isogenic parents, IRRR13 and IR24

Table 2 Marker genotypes of 18 recombinant individuals from the third F_2 population ($n = 1{,}972$) and their reactions to the *Xoo* strain PXO99

Individuals	Phenotype	Marker genotype					
		E6a	RP7	RP8	ST9	RP10	SR11
1B08	R	H	R	R	R	R	R
2E06	S	R	H	H	H	H	H
4C11	R	H	R	R	R	R	R
6A02	R	H	R	R	R	R	R
9B08	R	H	R	R	R	R	R
11G08	R	H	R	R	R	R	R
13B02	R	H	R	R	R	R	R
16A07	S	R	H	H	H	H	H
2D03	S	**R**	**R**	H	H	H	H
14C07	S	**R**	**R**	H	H	H	H
2A10	R	R	R	R	R	R	**H**
3F10	R	R	R	R	R	R	**H**
7D01	R	R	R	R	R	R	**H**
9G05	S	H	H	H	H	H	**R**
11A10	S	H	H	H	H	H	**R**
18B03	R	R	R	R	R	R	**H**
3A08	S	H	H	H	**R**	**R**	**R**
15B09	R	R	R	R	**S**	**S**	**S**

R, S and H stand for the homozygous resistant, homozygous susceptible and heterozygote genotypes, respectively, of the F_2 recombinants from the cross between the resistant and susceptible near-isogenic parents, IRRR13 and IR24

showed recombination between RP10 and *xa13* loci as well as between ST9 and *xa13* loci. Markers RP7 and RP8 co-segregated with *xa13* in the eight plants identified using marker SR11 (Table 2). The combined results obtained from the second and third F_2 populations led us to locate *xa13* to a single fragment flanked by RP7 and ST9. The sequence analyses of BAC clones 21H14 and 14L03 revealed that this fragment was only 14.8 kb in length (Fig. 1).

The *xa13* locus was reportedly flanked by RG136 and R2027 and mapped on the BAC clone, 21H14 (Sanchez et al. 1999). According to the sequence of marker R2027 (GenBank accession number: D24483), it locates between markers RP5 and SR6 in our map (Fig. 1). Thus, our results indicated that the *xa13* locus is found on BAC clone 14L03 instead of in 21H14 (Fig. 1). To confirm this result, the phenotypes and genotypes of four recombinant F_2 individuals (61C05, 2D03, 14C07 and 3A08) (Tables 1, 2), that define the fine location of the *xa13* locus, were further verified by examining the lesion length and marker genotypes of their F_3 progenies (more than ten F_3 plants from each of the F_2 individuals). After inoculation with the *Xoo* strain PXO99, no phenotypic segregation was observed within the F_3 plants from the resistant F_2 individual 61C05. The genotypes of the F_3 plants from 61C05 segregated in the expected ratio of approximately 1:2:1 (homozygous for the resistant IRBB13 allele:the heterozygote:homozygous for the susceptible IR24 allele, respectively) in the loci of E6a and RP7 ($\chi^2 = 0.88$, $P > 0.5$, Table 1). Also, plants in each F_3 family from the three susceptible F_2 individuals, 2D03, 14C07 and 3A08 (Table 2), segregated into susceptible and resistant plants. F_3 plants from 2D03 ($\chi^2 = 0.50$, $P > 0.75$) and 14C07 ($\chi^2 = 0.38$, $P > 0.75$) also co-segregated at RP8, ST9 and SR11 loci with the expected ratio of approximately 1:2:1 (homozygous for the resistant IRBB13 allele:the heterozygote:homozygous for the susceptible IR24 allele, respectively). F_3 plants from 3A08 co-segregated with E6a, RP7 and RP8 loci in the expected ratio of approximately 1:2:1 (homozygous for the resistant IRBB13 allele:the heterozygote:homozygous for the susceptible IR24 allele, respectively) ($\chi^2 = 0.14$, $P > 0.9$). These results further confirmed that the F_2 individuals used for the fine physical mapping of *xa13* were reliable.

Candidate genes of *xa13*

Analysis of the 14.8-kb sequence flanked by markers RP7 and ST9 revealed that this region contains two genes predicted by the combined use of FGENESH, GeneMark.hmm and GENSCAN. One of these two genes is a homologue of proline-rich extensin-like genes and the other is a homologue of the gene for nodulin MtN3. A third gene has only its 5′ end included in the 14.8-kb fragment. This gene remains an *Xa13* candidate, however, because the recombination studies map the site of the polymorphism that differentiates *Xa13* from *xa13*, and this could be a 5′ end sequence change (e.g., an altered promoter). However, this partially included third gene shows no significant BLAST homology to any sequences other than those found in rice. Hence, it is either a gene that is unique to rice or (more likely) is either a rapidly evolving transposable element or an annotation artifact (Bennetzen et al. 2004). The candidate extensin-like gene shows excellent (*E* value 10^{-23}) homology to an extensin-like gene in maize (accession Z34465). The putative MtN3 nodulin gene also exhibits very high homology to an NCBI protein database accession (no.

BAD13168, *E* value 10^{-114}) from rice. The BAC libraries used for the physical map were not from any of the lines used for the genetic mapping. However, all the markers that were developed based on the sequences of the BAC clones and used in the physical map were also found in the genetic analysis, indicating that the region does not differ greatly in gene content or other sequence content in different genetic backgrounds. Hence, the *Xa13* gene (dominant allele) is likely to be found on the sequenced BACs. This analysis indicated that *xa13* is a novel type of *R* gene that shows no sequence similarity with any known plant *R* genes. Functional verification of the *xa13* candidates is under way.

PCR-based markers for marker-assisted selection of *xa13*

As byproducts of our map-based cloning effort, a number of PCR-based markers for *xa13* were developed, which provide a useful tool for the marker-assisted transfer of this recessive *R* gene in rice breeding programs. Specifically, four PCR-based markers (E6a, SR6, ST9 and SR11) that were tightly linked to *xa13* should be more powerful and user-friendly than the previously identified RFLP markers (Zhang et al. 1996; Sanchez et al. 1999) because of their tight linkages with *xa13* and the expected high level of polymorphism in rice germplasm.

Conclusions

We have targeted a fully recessive gene, *xa13*, for bacterial blight resistance to a 14.8-kb DNA fragment. Four PCR-based markers that tightly flank the *xa13* locus were developed. These results will greatly facilitate the isolation and characterization of *xa13*, which appears to be a novel type of *R* gene in plants. Our work also provides useful information and powerful tools for marker-assisted transfer of *xa13* for combating bacterial blight disease in rice breeding programs worldwide.

Acknowledgements This research was supported by grants from the National Program of Research and Development of Transgenic Plants of China, National Special Key Program on Rice Functional Genomics, and the National Natural Science Foundation of China.

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