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Genetic and physical mapping of a new gene for bacterial blight resistance in rice

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Abstract The inheritance of resistance for bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), was studied in Minghui 63, an elite restorer line for a number of widely used rice hybrids in China. A new dominant gene against a Chinese *Xoo* strain JL691 in both the seedling and adult stages was identified in Minghui 63 and designated as *Xa26*(*t*). Using a total of 477 highly susceptible individuals from an F_2 population, the *Xa26*(*t*) locus was mapped to a region of about 1.68 cM. This locus co-segregated with marker R1506 and was 0.21 cM from marker RM224 on one side and 1.47 cM from marker Y6855RA on the other side, in rice chromosome 11. A contig map, composed of five non-redundant bacterial artificial chromosome (BAC) clones and spanning approximately 500 kb in length, was constructed. Analysis of recombination events in the *Xa26*(*t*) region with the highly susceptible F_2 individuals anchored the gene locus to a region covered by three overlapped BAC clones. Assay of the lines showing a double crossover in marker loci flanking *Xa26*(*t*), in a population of recombinant inbred lines carrying *Xa26*(*t*), further delineated the gene to a 20-kb fragment. The *Xa26*(*t*) locus is tightly linked to another bacterial blight resistance gene locus, *Xa4*.

Keywords Bacterial blight · *Xoo* · *R* gene · Mapping · Contig · Rice

Introduction

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most economically serious plant diseases worldwide. Chemical control has not been effective, and the development of resistant cultivars is considered to be the most effective way to control this disease. Currently, more than 20 genes conferring host resistance against various strains of *Xoo* have been identified, and these, have been designated in a series from *Xa1* to *Xa25*(*t*) (Lin et al. 1996; Nagato and Yoshimura 1998; Zhang et al. 1998; Khush and Angeles 1999; Chen 2002). Based on morphological and molecular markers, some of the resistance genes against bacterial blight have been mapped to rice chromosomes. Examples of the mapping include *Xa1, Xa2, Xa12* and *Xa14* on chromosome 4, *xa5* on chromosome 5, *Xa7* on chromosome 6, *xa13* on chromosome 8, *Xa3, Xa4, Xa10, Xa21, Xa22*(*t*) and *Xa23*(*t*) on chromosome 11 and *Xa25*(*t*) on chromosome 12 (Lin et al. 1996; Nagato and Yoshimura 1998; Zhang et al. 1998; Chen et al. 2002). The mapping of these resistance genes is exploited in marker-assisted selection in rice breeding programs. Pyramid lines incorporating two, three or four bacterial blight resistance genes show a higher and wider spectrum of resistance (Huang et al. 1997).

The mapping of bacterial blight resistance genes has also facilitated the generation of physical maps and, ultimately, the isolation of resistance genes. Currently, two recessive genes, *xa5* and *xa13*, and a dominant gene, *Xa4*, have been physically mapped (Yang et al. 1998; Sanchez et al. 1999; Sun et al. 2003). Two bacterial blight resistance genes, *Xa21* and *Xa1*, have been cloned (Song et al. 1995; Yoshimura et al. 1998). The products of the two genes have the recognizable structural domains carried by most of the known plant resistance genes. The *Xa21* encodes LRR (leucine-rich repeat)-kinase protein (Song et al. 1995) and the *Xa1* encodes NBS (nucleotide-binding site)-LRR protein (Yoshimura et al. 1998).

Minghui 63 is a restorer line widely used in hybrid rice production in China. The hybrids produced with Minghui 63 have many important virtues, such as high yield and wide adaptability, which enable these hybrids to account for more than 20% of the total rice production area in China for the last two decades. One of the important

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characteristics accounting for the wide adaptability of the hybrids produced with Minghui 63 is their resistance to bacterial blight, one of the most economically serious diseases of rice in China. A previous study indicated that Minghui 63 carries at least two bacterial blight resistance genes (Chen et al. 2002). The *Xa25*(*t*) gene, located on chromosome 12, was against a Philippine *Xoo* strain PXO339 at both the seedling and booting stages; this gene also showed incompatible reaction with a Chinese *Xoo* strain JL691 at seedling stage but not booting stage (Chen et al. 2002). Another bacterial blight resistance gene carried by Minghui 63 was dominantly resistant to JL691 at both seedling and booting stages; this gene was roughly mapped to the terminal region of the long arm of chromosome 11, where several bacterial blight resistance genes (e.g. *Xa3, Xa22*(*t*) and *Xa4*) have been localized (Yoshimura et al. 1995; Lin et al. 1996; Chen et al. 2002; Sun et al. 2003). However, the relationship of the gene against JL691 in Minghui 63 with other bacterial blight resistance genes located in the terminal region of chromosome 11 is unknown.

The objectives of the investigation reported here were: (1) to characterize and genetically fine-map the bacterial blight resistance gene on chromosome 11 in Minghui 63; (2) to construct a physical map encompassing the gene using bacterial artificial chromosome (BAC) libraries; (3) to identify the DNA fragment that contains the gene.

Materials and methods

Plant materials

Three segregation populations were used for identifying and mapping the bacterial blight resistance gene against *Xoo* strain JL691 in rice (*Oryza sativa* ssp. *indica*) cv. Minghui 63. The first population consisted of 2,533 F_2 individuals from a cross between cvs. Zhenshan 97 and Minghui 63. From this population, a total of 477 individuals highly susceptible to strain JL691 were obtained for fine genetic mapping of the gene. The second population included 241 recombinant inbred lines (RILs) developed by single seed descendent from a cross between Zhenshan 97 and Minghui 63 (Xing et al. 2002). Five lines of this population were used for physical mapping of the bacterial blight resistance gene. The third population consisted of 500 F_2 individuals developed from a cross between Zhenshan 97 and a line (R202) from the RIL population. R202 was identified by its molecular marker genotype as having the Zhenshan 97 (susceptible) allele at *Xa25*(*t*) locus and the Minghui 63 allele at the *Xoo* resistance gene region of chromosome 11, and thus was resistant to JL691 (Chen et al. 2002).

Xoo inoculation and disease scoring

A total of 12 *Xoo* strains were used for screening, including four Chinese strains, (KS-1-21, LN44, Zhe173 and JL691), two Japanese strains (T7133 and T7147) and six Philippine strains (PXO61, PXO86, PXO112, PXO99, PXO145 and PXO339). Plants were inoculated at the booting stage (approximately 40 days after transplanting) using the leaf-clipping method (Kauffman et al. 1973) on ten of the uppermost fully expanded leaves of each plant. The preparation of bacterial inoculum was as described by Lin et al. (1996). Mock-inoculated (control) plants were treated under the same conditions except that pathogen inoculum was replaced by water. The disease was scored 2–3 weeks after inoculation by measuring the lesion length.

Fine genetic mapping of bacterial blight resistance gene

The RFLP (restriction fragment length polymorphism) and SSR (simple sequence repeat) markers covering the terminal region of chromosome 11 were selected for fine genetic mapping of the bacterial blight resistance gene in Minghui 63. The linkage relationship between the target gene and the molecular markers was analyzed using 477 highly susceptible individuals of the F_2 population developed from a cross between Zhenshan 97 and Minghui 63. The recombination frequencies were calculated using the maximum likelihood estimator (Allard 1956): $c = (N_1 + N_2/2)/N$, where N is the total number of susceptible plants surveyed, N_1 is the number of individuals homozygous for the banding patterns of resistant parent and N_2 is the number of individuals heterozygous for the banding patterns of the two parents. The variance was given by $Vc = c\ (1 - c)/2N$.

Physical mapping of bacterial blight resistance gene

Two BAC libraries were used for physical mapping of the resistance gene. One library with average insert length of 150 kb and ninefold coverage of rice haploid genome was constructed from Minghui 63 (Peng et al. 1998). The other library constructed from Nipponbare (*Hin*dIII) was kindly provided by Dr. R. Wing, Clemson University. All candidate BAC clones identified with markers in the region containing the gene locus were subjected to Southern hybridization analysis to confirm their overlapping relationship and the relationship with linked markers. The insert size of each BAC clone was determined by pulse-field gel electrophoresis after digestion with restriction enzyme *Not*I. Subclones of the BACs covering the gene locus were used as markers for further fine physical mapping of the gene.

Results

Phenotypic and genetic analysis

A total of 2,533 F_2 individuals from the cross between Zhenshan 97 and Minghui 63 were inoculated with *Xoo* strain JL691 at the booting stage. Five hundreds individuals were randomly chosen for measuring the lesion length. The distribution of the lesion length in the 500 plants was bimodal with an apparent valley at approximate 3 cm (Fig. 1). Using a lesion length of 3 cm as the dividing point, the numbers of resistant (lesion length

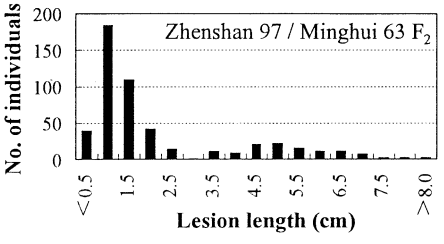

Fig. 1 The distribution of lesion length after inoculation with *Xoo* strain JL691 in a sample containing 500 randomly individuals from a F_2 population developed from a cross between Zhenshan 97 and Minghui 63. The average lesion lengths of Minghui 63 and Zhenshan 97 were 0.5 cm and 11.5 cm, respectively

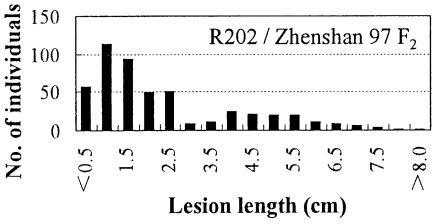

Fig. 2 The distribution of lesion length after JL691 inoculation in a F_2 population of a cross between R202 and Zhenshan 97. The average lesion lengths of R202, Zhenshan 97 and Minghui 63 were 0.8, 11 and 0.5 cm, respectively

< 3 cm) and susceptible (lesion length ≥ 3 cm) F_2 individuals were 388 and 112, respectively, which fit the expected 3:1 ratio ($\chi^2 = 1.802$, $P > 0.1$), indicating that the resistance of Minghui 63 was controlled by a single dominant gene.

To confirm that *Xa25*(*t*) located on chromosome 12 was not involved in the resistance of Minghui 63 to strain JL691 at the booting stage, 500 F_2 individuals from a cross between R202 and Zhenshan 97 were inoculated with JL691 at the booting stage. R202, which is resistant to JL691, had the Minghui 63 allele in the region of chromosome 11 putatively containing resistance gene against JL691 and had the Zhenshan 97 (susceptible) allele at the *Xa25*(*t*) locus (Chen et al. 2002). The distribution of lesion length in this F_2 population was also bimodal with an apparent valley at approximately 3 cm (Fig. 2). The numbers of resistant (372 with lesion length ≤ 3 cm) and susceptible (128 with lesion length > 3 cm) individuals fit the expected 3:1 ratio ($\chi^2 = 0.096$, $P > 0.5$). The results suggested that the resistance of Minghui 63 to strain JL691 at the booting stage was conferred by the gene located on chromosome 11.

To examine the relationship of the resistance gene against *Xoo* strain JL691 in Minghui 63 with the known bacterial blight resistance genes, *Xa3, Xa4, Xa10* and *Xa22*(*t*), all located in the terminal region of chromosome 11, we tested resistant lines carrying these genes with a set of *Xoo* strains representing wide spectrum of virulence. This included a set of near-isogenic lines each carrying a different bacterial blight resistance gene – IRBB3 (*Xa3*), IRBB4 (*Xa4*) and IRBB10 (*Xa10*) – and their recurrent parent, IR24, as well as cvs. Zhachanglong [*Xa22*(*t*)], Minghui 63, R202 and Zhenshan 97. Minghui 63 and R202 showed a similar spectrum of resistance against the set of *Xoo* strains and differed only in the resistance against PXO339 (Table 1), suggesting that R202 did not carry *Xa25*(*t*) from Minghui 63. The resistant spectrum of the other tested germplasms, carrying *Xa3, Xa4, Xa10* and *Xa22*(*t*), respectively, were distinctively different from Minghui 63 and R202 (Table 1), indicating that the gene against strain JL691 in Minghui 63 and R202 was new in the sense that its gene identity has not been determined previously. We tentatively designate it as *Xa26*(*t*).

Fine genetic mapping of the *Xa26*(*t*) gene

Based on the preliminary mapping result of the resistance gene against JL691 in Minghui 63 (Chen et al. 2002), molecular markers located on the terminal region of the long arm of chromosome 11 were used to screen the 477 highly susceptible individuals (lesion length ≥ 4 cm) of the F_2 population developed from a cross between Zhenshan 97 and Minghui 63. Genetic distances between the *Xa26*(*t*) locus and nearby markers were calculated. The *Xa26*(*t*) locus co-segregated with RFLP marker R1506 and was 0.21 cM from a SSR marker, RM224, on one side and 1.47 cM from two co-segregating RFLP markers, Y6855RA and S12886, on the other side (Fig. 3).

To take a candidate gene approach to the *Xa26*(*t*) locus, a rice clone, NBS119, that had been previously mapped to this chromosomal region (M.A. Saghai Maroof

Table 1 Performance[a] of rice cultivars after inoculation with *Xoo* strains

Xoo strain	Booting stage							
	ZS97[b]	MH63[b]	R202	ZCL[b]	IRBB3	IRBB4	IRBB10	IR24
Chinese strains								
KS-1-21	S	S	S	MR	S	R	S	S
LN44	S	MR	MR	R	R	MR	S	S
Zhe173	S	S	S	MR	MR	R	S	S
JL691	S	R	R	R		MR	R	S
Japanese strains								
T7133	S	MR	MR	R	MR	MS	S	S
T7147	S	MR	S	R	R	MS	S	S
Philippine strains								
PXO61 (race 1)	S	MR	MR	R	R	R	S	S
PXO86 (race 2)	S	MS	MS	R	R	S	R	S
PXO112 (race 5)	S	S	S	R	R	R	R	S
PXO99 (race 6)	S	S	S	R	S	S	S	S
PXO145 (race 7)	S	MR	MS	R	R	R	R	S
PXO339 (race 9)	S	R	S	R	R	S	S	S

[a] R, Resistant; MR, moderately resistant; S, susceptible; MS, moderately susceptible
[b] ZS97, Zhenshan 97; MH63, Minghui 63; ZCL, Zhachanglong

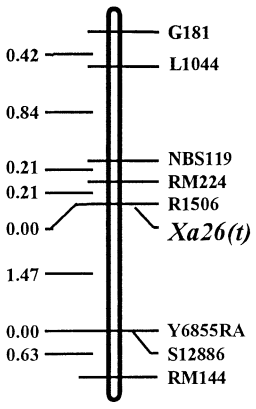

Fig. 3 The location of bacterial blight resistance gene *Xa26*(*t*) on the molecular linkage map of chromosome 11

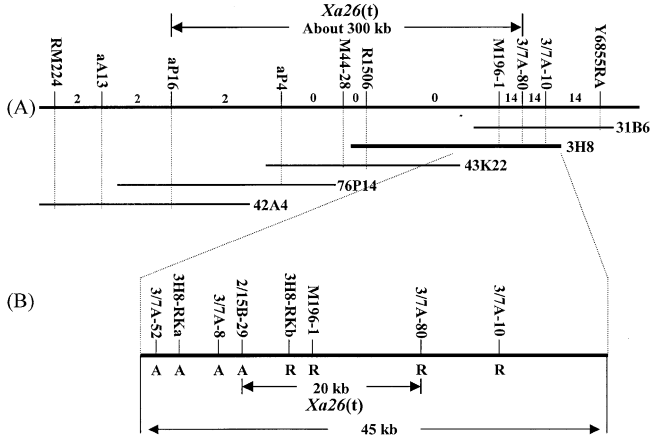

Fig. 4 A A contig map covering the *Xa26*(*t*) region was constructed with 16 recombinants from the highly susceptible sample of the F_2 population of a cross between Zhenshan 97 and Minghui 63. The *long horizontal line* indicates the genomic region containing the *Xa26*(*t*) gene. The *short horizontal lines* represent BAC clones. aA13 and aP16 were subclones from Nipponbare BAC clone 42A4-aP4 and M44-28 were subclones from Nipponbare BAC clones 76P14 and 43K22, respectively; M196-1, 3/7A-80 and 3/7A-10 were subclones from Minghui 63 BAC clone 3H8. The *numbers* between molecular markers indicate the numbers of recombination events detected between the *Xa26*(*t*) locus and corresponding molecular marker. The *dashed vertical lines* between the markers and BAC clones indicate that hybridization between marker and BAC clone(s) was verified. **B** The fine regional physical map of BAC clone 3H8 was constructed with five resistant RILs (R85, R96, R102, R117 and R240) from a cross between Zhenshan 97 and Minghui 63, showing recombination events at both sides of *Xa26*(*t*) locus. The *capital letters under* the names of the subclones from BAC clone 3H8 indicate that a subclone detected the band pattern of the resistant (*R*) parent (Minghui 63) or the abnormal (*A*) (not Minghui 63 nor Zhenshan 97) band pattern in the five lines

et al., unpublished data) and homologous to the NBS motif of plant disease resistance genes was used as probe to test its co-segregation with *Xa26*(*t*). The NBS119 did not co-segregate with *Xa26*(*t*) and was mapped between markers L1044 and RM224 (Fig. 3).

The 2 and 20 susceptible F_2 recombinants screened out by RM224 and RM144, respectively, and used for fine genetic mapping of *Xa26*(*t*) were verified through examining the phenotypes and genotypes of their F_3 families. The F_3 progenies were tested for reactions to JL691 and assayed with SSR markers RM224 and RM144. As expected, all of the individuals in all the F_3 families were highly susceptible; segregation was observed at the RM224 locus for the progenies of two F_2 recombinants and at the RM114 locus for the progenies of the other 20 F_2 recombinants. Thus, the verification of the 22 recombinants further confirmed the mapping result.

Physical mapping of the *Xa26*(*t*) gene

Based on the physical map spanning the bacterial blight resistance gene *Xa4*, there were eight BAC clones from the Minghui 63 BAC library encompassing the genomic region between R1506 and Y6855RA (Fig. 3) (Sun et al. 2003). A fingerprint map of rice constructed with Nipponbare BAC clones released on the web site of the Clemson University (http://www.clemson.edu) was used to construct the contig covering the genomic region between R1506 and RM224 (Fig. 3). According to the fingerprint map by Clemson University, 20 BAC clones covering the genomic region between L1044 and R1506 were picked out from the library and screened with the markers flanking the *Xa26*(*t*) locus (Fig. 3). One marker, RM224, was anchored to BAC clone 42A4 and another, R1506, was anchored to BAC clone 43K22. The overlapping relationship among different BAC clones from the Nipponbare BAC library was confirmed by Southern hybridization analysis. The relationship between the contig constructed with the Minghui 63 BAC clones and the contig constructed with the Nipponbare BAC clones was also examined by Southern hybridization (data not shown). Finally, a contig encompassing the *Xa26*(*t*) locus was formed that consisted of five BAC clones with minimal redundance. Of the five BAC clones, 31B6 and 3H8 were from the Minghui 63 BAC library and 43K22, 76P14 and 42A4 from the Nipponbare BAC library. This contig covered the genomic region between RM224 and Y6855RA and spanned approximately 500 kb in length (Fig. 4A).

Sixteen F_2 individuals showing recombination events between the *Xa26*(*t*) locus and the flanking markers RM224 and Y6855RA from the 477 highly susceptible plants were used to reduce the region containing *Xa26*(*t*). Subclones from the BAC clones covering the *Xa26*(*t*) region were used to screen these recombinants. The *Xa26*(*t*) locus co-segregated with aP4, M44-28 and M196-1 from BAC clones 76P14, 43K22 and 3H8, respectively (Fig. 4A). Two recombinants were detected by aP16, a subclone of BAC 42A4 from one side of the *Xa26*(*t*) locus. The subclone 3/7A-80 of BAC 3H8 identified 14 recombinants from another side of the *Xa26*(*t*) locus. The subclone aP16 also hybridized with BAC clone 76P14. Thus, the *Xa26*(*t*) locus was reduced to a region covered by three BAC clones, 76P14, 43K22 and 3H8 (Fig. 4A).

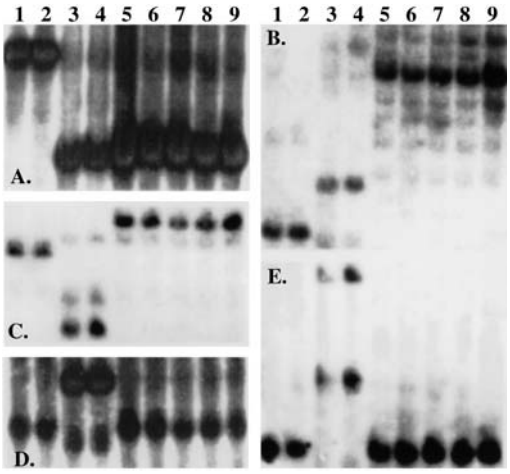

Fig. 5A–E Southern blot analysis of total DNA from the lines of the RIL population. *Lanes 1*: resistant parent, Minghui 63, *2* susceptible parent, Zhenshan 97, *3, 4* the lines (R202 and R59) showing a corresponding band pattern as its resistant or susceptible phenotype, respectively, *5–9* the lines (R85, R96, R102, R117 and R240) having the abnormal (not Minhui 63 nor Zhenshan 97) band pattern in the *Xa26*(*t*) region. **A** DNA digested with *Bgl*II and hybridized with R1506, **B** DNA digested with *Eco*RV and hybridized with 3/7A-52, **C** DNA digested with *Hin*dIII and hybridized with 2/15B-29, **D** DNA digested with *Hin*dIII and hybridized with 3H8-RKb, **E** DNA digested with *Dra*I and hybridized with 3/7A-80

Five RILs, R85, R96, R102, R117 and R240, from a cross between Zhenshan 97 and Minghui 63 that showed recombination events at both sides of the *Xa26*(*t*) locus were used for further reducing the region containing the locus. All the five lines were resistant to JL691 but had alleles from the susceptible parent at marker locus R1506 that co-segregated with *Xa26*(*t*) and also at RM224 and RM114 flanking *Xa26*(*t*) on both sides (Figs. 3, 5). We then used eight subclones from BAC clone 3H8 as probes to screen the five RILs: four subclones, 3H8-RKb, M196-1, 3/7A-80 and 3/7A-10 (GenBank accession no: AY150307), detected alleles from the resistant parent (Minghui 63), and the other four subclones, 2/15B-29, 3/7A-8, 3H8-RKa and 3/7A-52 (GenBank accession no. AY150306), revealed abnormal banding patterns that were not present in either of the parents (Figs. 4B, 5). Combining the results obtained from the analysis of 16 F_2 recombinants, the region containing *Xa26*(*t*) gene was further narrowed down to a fragment flanked by 2/15B-29 and 3/7A-80 (Fig. 4). Sequence analysis of BAC clone 3H8 (data will be presented elsewhere) revealed that this fragment was 20 kb in length.

Discussion

The present study identified and located a new dominant gene, *Xa26*(*t*), for bacterial blight resistance on the terminal region of the long arm of chromosome 11 in rice cv. Minghui 63. This new gene showed an incompatible reaction with a Chinese *Xoo* strain JL691 at both the seedling and adult stages. The physical localization of this gene to a 20-kb DNA fragment represented a significant step toward the final goal of cloning this gene. A number of DNA markers tightly linked to *Xa26*(*t*) will also facilitate marker-assisted selection of this new gene in rice breeding programs.

Several bacterial blight resistance genes, *Xa3, Xa4* and *Xa22*(*t*), have previously been mapped to the region containing *Xa26*(*t*) (Yoshimura et al. 1995; Lin et al. 1996; Sun et al. 2003). Two common markers, 2/15B-29 and M196-1, with the latter toward the end of chromosome 11, were used in this study and in fine physical mapping of *Xa4* (Sun et al. 2003). The M196-1 marker co-segregated with *Xa26*(*t*), and recombinants were detected between the 2/15B-29 and the *Xa26*(*t*) loci (Fig. 4). However, the 2/15B-29 marker co-segregated with *Xa4*, and one recombinant was detected between *Xa4* and M196-1 (Sun et al. 2003). Thus, the *Xa4* and *Xa26*(*t*) loci are tightly linked with the *Xa26*(*t*) locus in the terminal region of the chromosome. The *Xa4* locus is also tightly linked to the *Xa3* locus (Yoshimura et al. 1995). Therefore, the *Xa26*(*t*) locus may be allelic or tightly linked to the *Xa3* locus. Fine genetic mapping of the *Xa22*(*t*) gene showed that *Xa22*(*t*) co-segregated with RFLP marker R1506 (Wang 1999). The same marker also co-segregated with *Xa26*(*t*) in this study (Fig. 3). Thus, it can be speculated that the *Xa26*(*t*) locus is also tightly linked or allelic to the *Xa22*(*t*) locus.

Plant disease resistance genes frequently occur in the form of a tandem repeated multi-gene family. A striking example of rice is the bacterial blight resistance gene *Xa21* family on chromosome 11, which is composed of at least eight members situated within a 230-kb region. The seven members, other than *Xa21* itself, appear to have completely or partially lost resistance to *Xoo* (Song et al. 1997; Wang et al. 1998). The close linkage of *Xa26*(*t*) locus with *Xa3, Xa4* and *Xa22*(*t*) suggests that these resistance genes may belong to a multi-gene family.

The rice genetic linkage map is about 1,522 cM (Harushima et al. 1998). The genome size of rice is about 430 Mb (Arumuganathan and Earle 1991). Thus, the ratio of physical and genetic distances of the rice genome is approximately 280 kb/cM. The ratio of physical and genetic distance varied largely in the region containing *Xa26*(*t*) locus (Fig. 4). The 0.21-cM region flanked by aP16 and R1506 corresponded to a physical distance 200 kb in length, which is converted to approximately 1,000 kb/cM; this is three to four times greater than the average ratio between physical and genetic distances of the rice genome. However, a ratio of less than 100 kb/cM was observed in the region covered by BAC clone 3H8. Apparently, some hot spots of crossovers exist in this 100-kb region. Such a big difference in the ratios of physical to genetic distances in very closely located neighboring fragments was also observed in the fine genetic and physical mapping of the *Xa4* gene (Sun et al. 2003). In addition to the tight linkage to or allelic with *Xa3, Xa4* and *Xa2*(*t*), the *Xa26*(*t*) locus also appears to be closely linked to two loci for blast resistance, *Pi1* and *Pi44*(*t*)

(Mew et al. 1994; Yu et al. 1996; Chen et al. 1999). The high frequency of recombination in resistance gene rich region may be the cause for the generation of new resistance genes.

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