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Identification of a 47-kb DNA fragment containing *Xa4*, a locus for bacterial blight resistance in rice

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Abstract Bacterial blight caused by *Xanthomonas oryzae* pv *oryzae* is a devastating disease in rice worldwide. The resistance gene *Xa4* has been widely used in breeding programs and played an important role in protecting rice from this disease. Using 642 highly susceptible individuals and a random sample of 255 individuals from an F_2 population developed from a cross between IRBB4 and IR24, the *Xa4* gene was genetically mapped to a region less than 1 cM. A contig map was constructed for the *Xa4* region consisting of six non-redundant bacterial artificial chromosome (BAC) clones and spanning approximately 500 kb in length. Analysis of recombination events in the *Xa4* region located the gene locus to one BAC, 3H8. Assay of the recombinants using the subclones of 3H8 in combination with sequence analysis further narrowed the *Xa4* locus down to a 47-kb fragment.

Keywords *R* gene · BAC contig · Mapping · Bacterial blight · *Xoo* · Rice

Introduction

Bacterial blight caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is a serious disease of rice. It is not only widespread throughout Asia but is also reported to occur in Australia, the United States and several rice-growing countries of Latin America and Africa. Yield loss due to the disease ranges from 20 to 30% (Ou 1985). The best control for bacterial blight is the use of varietal resistance. Currently, more than 20 resistance (*R*) genes against bacterial blight have been identified (Kinoshita 1994, 1995; Lin et al. 1996; Zhang et al. 1998; Gnanamanickam et al. 1999) and two of the *R* genes, *Xa21* and *Xa1*, have been isolated (Song et al. 1995; Yoshimura et al. 1998). In addition, a number of the *R* genes against bacterial blight have been incorporated into improved rice varieties, which are now widely grown in many rice-producing countries.

The dominant gene *Xa4* has been widely used in breeding programs in Asia and played an important role in protecting rice from *Xoo*. In China, almost all of the commercial hybrids and conventional cultivars of *indica* rice in the main rice-growing regions contain this gene (Min 1992). However, as with many other plant resistance genes identified to-date, the mechanism of resistance conferred by the expression of *Xa4* has remained unknown. Identifying the DNA sequence of this gene should provide clues to the mechanism for resistance of the gene.

Xa4 was first identified by Petpisit et al. (1977). Yoshimura et al. (1995) mapped the gene roughly to the terminal region of chromosome 11. Li et al. (1999) further mapped the *Xa4* locus between two RFLP (restriction fragment length polymorphism) markers, RZ536 and L457b. Wang et al. (2001) localized the *Xa4* locus between G181 and L1044 at a distance of 4.4 and 3.8 cM from the flanking markers, respectively. However, using other rice molecular-marker linkage maps as references (Harushima et al. 1998; Wang 1999), it can be deduced that the *Xa4* locus reported by Li et al. (1999) and Wang et al. (2001) was not in the same position.

The objectives of this study were: (1) to genetically fine-map the *Xa4* locus using populations derived from a large number of field-grown F_2 individuals, (2) to construct a contig map for the genomic region containing the *Xa4* locus using clones from a BAC (bacterial artificial chromosome) library, and (3) to localize the *Xa4* locus to a delineated DNA fragment.

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Materials and methods

Mapping population and disease evaluation

Two thousands and eight hundred F_2 individuals were obtained from a cross between a bacterial blight-resistant near-isogenic line IRBB4 (*Oryza sativa* ssp. *indica*) (Yoshimura et al. 1995), containing bacterial blight resistance gene *Xa4* and its susceptible recurrent parent, IR24 (*indica*). At the booting stage, five of the uppermost fully expanded leaves of each plant were inoculated with Philippine race 1 (PXO61) of *Xoo* by the leaf-clipping method (Kauffman et al. 1973) in a disease nursery. Reaction to the pathogen was evaluated 21 days after inoculation by measuring the average lesion length of three leaves.

DNA extraction and hybridization

Total cellular DNA was extracted by the CTAB method (Murray and Thompson 1980). DNA digestion, Southern blotting and hybridization were conducted according to the procedures described previously (Liu et al. 1997). After hybridization, filters were washed in 1 × SSC and 0.1% SDS once for 5 min at room temperature and for 10 min at 65 °C.

Construction of the physical map of the *Xa4* region

A BAC library constructed with genomic DNA from rice cultivar Minghui 63 (*indica*) with average insert length of 150 kb and a coverage equivalent to nine genomes (Peng et al. 1998) was used for the construction of the physical map. The insert sizes of BAC clones covering the *Xa4* region were determined by pulse field gel-electrophoresis (PFGE) after digestion with restriction enzyme *Not*I. The conditions for PFGE were as follows: switch time ramping from 1 to 12 s, temperature 11 °C, 6 V/cm, pulse angle 120°, using 0.5 × TBE buffer for 12 h. The size of the BAC clone was estimated based on its migration as compared to the standard high-molecular-weight DNA marker.

Cloning of the homologous sequences of *R* genes

The polymerase chain reaction (PCR) method was applied to amplify *R* gene homologues from BAC clones covering the *Xa4* region. Degenerated primers complementary to the two highly conserved amino-acid domains, GGVGKTT and GLPLAL, in the NBS (nucleotide-binding site) motif of known *R* genes (Bent et al. 1994; Whitham et al. 1994; Grant et al. 1995; Lawrence et al. 1995) were used for PCR. The primer sequences kindly provided by Dr. S. H. Hulbert (Kansas State University), were 5′-GGI GGI GTI GGI AAI ACI AC-3′ (forward) and 5′-ARI GCT ARI GGI ARI CC-3′ (reverse) (R, representing a mixture of bases A and G; I, inosine). The DNA was amplified at 94 °C for 1 min, 42 °C for 1 min and 72 °C for 1 min for a total 35 cycles. The PCR products were cloned into pGEM-T vector (Promega, USA) according to the manufacturer's specification.

Data analysis

To determine the linkage relationship between the *Xa4* locus and molecular markers, a two-step analysis was adopted. First, data obtained from 255 randomly chosen individuals of the F_2 population were analyzed with the Mapmaker/Exp 3.0 program at a LOD threshold of 3.0 (Lincoln et al. 1992) to construct a genetic map around the *Xa4* locus. Second, the markers located near the *Xa4* locus were used to assay 642 highly susceptible F_2 individuals for fine genetic and physical mapping of *Xa4*. The genotypes of the recombinants of the F_2 individuals with respect to the *Xa4* resistance gene and its flanking molecular markers were further verified by analyzing the segregation of the resistance and the markers in the respective F_3 families.

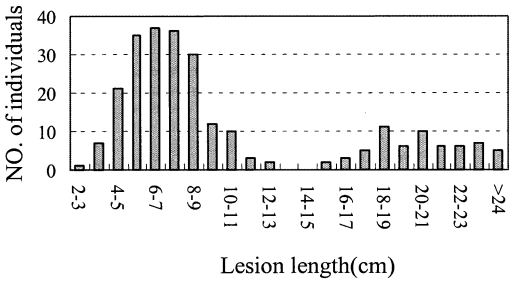

Fig. 1 Distribution of lesion length after PXO61 inoculation in a sample containing 255 randomly selected individuals from a F_2 population of a cross between IRBB4 and IR24

Results

Genetic mapping of the *Xa4* gene

The bacterial blight resistance gene *Xa4* is incompatible to *Xoo* strain PXO61 (Yoshimura et al. 1995). One of the parents of the F_2 population, IRBB4 carrying *Xa4*, was resistant to PXO61 with an average lesion length of 4.9 cm at 3 weeks after inoculation. The other parent of the population, IR24, was highly susceptible to PXO61 with an average lesion length of 22.4 cm. Two hundred and fifty five individuals randomly chosen from the F_2 population were used for genetic mapping of *Xa4*. The distribution of lesion length for PXO61 inoculation in the 255 F_2 plants was bimodal with an apparent valley at 13 to 15 cm (Fig. 1). When the F_2 individuals with a lesion length longer than 15 cm were classified as susceptible and those with a lesion length shorter than 13 cm were classified as resistant, the numbers of resistant and susceptible individuals fit the expected 3:1 ratio ($\chi^2 = 0.32$, $P > 0.5$), indicating that the resistance of IRBB4 to PXO61 was only controlled by *Xa4*.

The *Xa4* gene was previously mapped at the end of the long arm of chromosome 11 (Yoshimura et al. 1995; Li et al. 1999; Wang et al. 2001). The markers flanking the *Xa4* locus were chosen for fine genetic mapping of the gene in the present study. Six RFLP markers (G181, R1506, S12886, L190, C10295 and S10559) and one SSR (simple sequence repeat) marker (RM224) detected polymorphism between the parents of the F_2 population. Using the 255 F_2 individuals as the mapping population, the *Xa4* gene, as measured by resistance against *Xoo* strain PXO61, was mapped between two RFLP markers, R1506 and S12886, at a distance of 0.5 cM from both markers (Fig. 2).

To adopt a candidate gene approach to the *Xa4* locus, BAC clones from the Minghui 63 BAC library that partially covered the terminal region of chromosome 11 (Peng et al. 1998) were used as a template to amplify an NBS-like sequence of *R* genes. A 495-bp PCR product

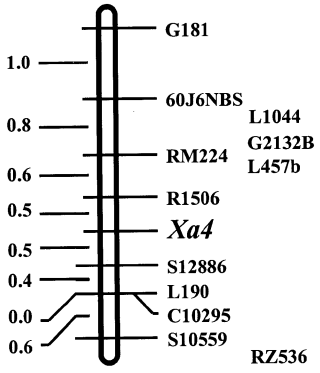

Fig. 2 The location of bacterial blight resistance gene *Xa4* on the molecular linkage map of chromosome 11. The positions of marker loci (L1044, G2132B, L457b and RZ536) placed on the right side of the map are deduced using information from the maps of Harushima et al. (1998), Li et al. (1999) and Wang (1999)

from the BAC clone 60J6, 60J6NBS (GenBank accession number: AF465253), was cloned. BLASTX analysis (Altschul et al. 1997) showed that the predicted product of 60J6NBS was highly homologous to the NBS-LRR-type resistance protein of rice (score = 299, E value = 6e-81) (Leister et al. 1998). However, 60J6NBS did not co-segregate with *Xa4* and was mapped between markers G181 and RM224 (Fig. 2). This clone was hence discounted as the candidate for *Xa4*.

Fine genetic and physical mapping of *Xa4* gene

Molecular markers flanking the *Xa4* locus were used to screen the Minghui 63 BAC library for the construction of the physical map covering the gene locus. Four overlapping BAC clones from the Minghui 63 BAC library were previously identified by marker R1506, which was found to be tightly linked to the *Xa4* locus in the present study (Fig. 2) (Peng et al. 1998). Marker S12886, located on the other side of the *Xa4* locus, screened out two overlapping BAC clones from the library. Thus two separated BAC contigs were obtained in the region containing *Xa4*. Two RFLP markers, Y6855RA and C481S cosegregating with S12886 (Harushirma et al. 1998), were also used to screen the library, resulting in four and three positive clones, respectively. All of the 13 clones identified by the above four RFLP markers were digested with restriction enzyme *Hin*dIII and transferred to a nylon filter. The filter was sequentially hybridized with the markers that identified the positive clones and also the BAC clones included in the filter, to determine the overlapping relationship of the various BAC clones (Fig. 3). Finally, a contig consisting of six minimum overlapping BAC clones and spanning approximately 500 kb in length was formed covering the *Xa4* region (Fig. 4).

For fine genetic and physical mapping of *Xa4*, 642 susceptible individuals (lesion length longer than 15 cm) from the F_2 population were used to identify recombinant events between the *Xa4* locus and tightly linked markers. Two SSR markers, RM224 and RM144 flanking *Xa4* (Temnykh et al. 2000), were first used to screen the 642 susceptible individuals. Three recombinant individuals (108, 21 and 5-13) were identified by RM224, and another three recombinants (10-11, 16-5 and 4-17) were identified by RM144 (Table 1). The genotypes of the six susceptible recombinant individuals were further examined using R1506 and S12886 as probes. R1506

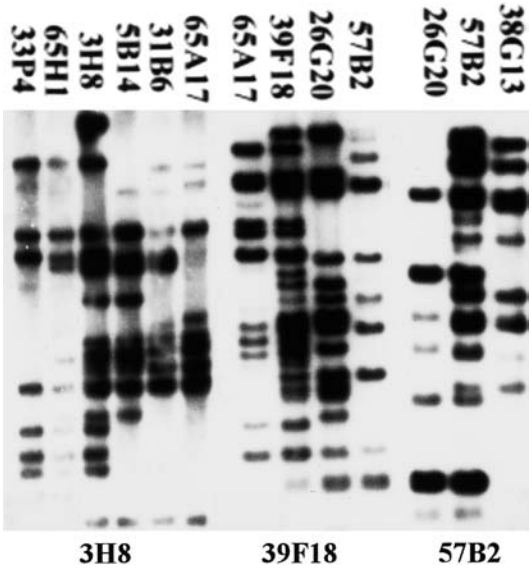

Fig. 3 Southern-blot analysis of *Hin*dIII-digested BAC clones covering the *Xa4* region. The filters were hybridized with 3H8, 39F18 and 57B2 digested with *Hin*dIII, respectively

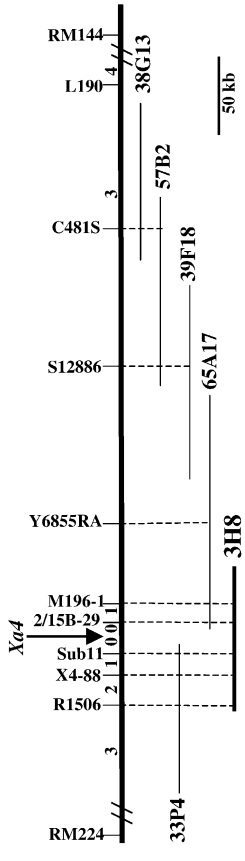

Fig. 4 A contig map covering the *Xa4* region. R1506, Y6855RA, S12886 and C481S were RFLP markers. X4-88, Sub11, 2/15B-29 and M196-1 were subclones from BAC clone 3H8. The *numbers* between molecular markers indicate the numbers of recombination events detected between the *Xa4* locus and corresponding molecular marker. The *long horizontal line* indicates the genomic region containing the *Xa4* gene. The *short horizontal lines* represent BAC clones. The *dashed vertical lines* between the markers and BAC clones indicate that hybridization between the marker and BAC clone(s) was verified

Table 1 Molecular marker genotypes of seven F_2 recombinant individuals

Marker	Individuals[a]						
	108	21	5-13	39	10-11	16-5	4-17
RM224	H	H	H	H	S	S	S
R1506	S	H	H	H	S	S	S
X4-88	S	S	H	H	S	S	
Bgl6-49	S	S	S	H	S	S	
Sub11	S	S	S	H	S	S	S
2/15B-29	S	S	S	H	S	S	S
M196-1	S	S	S	S	S	S	
S12886	S	S	S	S	S	S	S
L190	S	S	S	S	H	H	S
RM144	S	S	S	S	H	H	H

[a] S, homozygous for the allele from IR24; H, heterozygous for alleles from both parents.
The individual numbered 39 was resistant, and the other six individuals were susceptible

identified two of the three recombinant individuals as detected by RM224 indicating that R1506 is closer to *Xa4* than RM224, whereas no recombinant was detected between S12886 and the *Xa4* locus in the six susceptible individuals (Table 1). However, a resistant individual (39) was observed to be a recombinant between S12886 and *Xa4* in the sample of 255 random individuals. The same recombinant was also detected by RM144, a dominant marker Y6855RA and a previously obtained subclone (M196-1) of the BAC clone 3H8 (Wang 1999) (Table 1 and Fig. 4). According to the corresponding relationship of markers and BAC clones used for fine genetic and physical mapping of *Xa4*, it can be deduced that the *Xa4* locus is located between R1506 and M196-1 (Fig. 4). In other words, the BAC clone 3H8, about 100 kb in size, contained the *Xa4* locus.

The phenotypes and genotypes of the six susceptible and one resistant recombinant individuals identified from fine genetic and physical mapping of *Xa4* (Table 1) were further verified by examining the lesion length and marker genotypes of the respective F_3 progenies. After inoculation with PXO61, no phenotypic segregation was observed within each of the F_3 families of the six susceptible F_2 individuals. As expected, segregation was observed at the RM224 locus for the progenies of three susceptible individuals (108, 21 and 5-13) and at the RM144 locus for the progenies of the other three susceptible individuals (10-11, 16-5 and 4-17) (data not shown). Whereas, individuals in the F_3 family from the resistant F_2 individual (39) segregated into susceptible and resistant plants (data not shown). These F_3 plants also segregated at the RM224 locus, but not at the RM144 locus. These results further confirmed that the seven individuals used for fine genetic and physical mapping of *Xa4* were reliable.

To further reduce the genomic region containing the *Xa4* locus, three subclones of the BAC clone 3H8, X4-88 (GenBank accession number: AF521904), Sub11 (GenBank accession number: AF521905) and 2/15B-29, were used to assay the seven recombinant individuals. One recombinant event was detected between X4-88 and the *Xa4* locus and no recombination was detected between Sub11, 2/15B-29 and *Xa4* (Fig. 4). Thus the genomic region containing the *Xa4* locus was further narrowed down to the fragment bounded by X4-88 and M196-1. Sequence analysis of BAC clone 3H8 (data will be presented elsewhere) indicated that the distance between X4-88 and M196-1 was approximately 47 kb in length.

Discussion

The main accomplishment of this study is the genetic fine mapping and physical delineation of the *Xa4* locus to a DNA fragment of less than 47 kb in length. This result should be very useful for cloning the *Xa4* gene, which is now in progress. The close linkage of the gene locus with flanking molecular markers should also be very useful for transferring the gene in rice breeding programs.

The *Xa4* gene was previously mapped to the terminal region of chromosome 11 by several groups (Yoshimura et al. 1995; Li et al. 1999; Wang et al. 2001). Because no common flanking markers around the *Xa4* region were used between the present study and the study reported by Yoshimura et al. (1995), the mapping results in the two studies could not be compared directly. However, using other published molecular linkage maps of chromosome 11 as references (Harushima et al. 1998; Wang 1999), it is obvious that the *Xa4* locus determined in the present study was consistent with that reported by Li et al. (1999) who, using a mapping population developed from the parents different from the two used in the present study, located the gene between markers L457b and RZ536 (Fig. 2). However, the results obtained in the present study are not consistent with those reported by Wang et al. (2001), who mapped the *Xa4* locus between markers G181 and L1044, a region slightly different from the *Xa4* locus determined in the present study.

The ratio of the physical to genetic distance of the rice genome is about 260 kb per cM on average (Wu and Tanksely 1993). The present results showed that the ratio of physical to genetic distance was not uniform across the *Xa4* region. The physical distances were less than 90 kb between R1506 and M196-1 and more than 350 kb between R1506 and C481S. Interestingly, all crossovers occurred in the 90-kb region flanked by R1506 and M196-1, and none was found in the remainder of the 350-kb region. This suggested that there were some hotspots of crossovers in the 90-kb region. This phenomenon was also observed in other mapping populations, in which Y6855RA, S12886, C481S and several other markers cosegregated (Harushirma et al. 1998; Saji et al. 2001). Besides *Xa4*, the bacterial blight resistance gene *Xa22*(t) (Wang 1999) and another new bacterial blight resistance gene (Z. Yang et al., unpublished work) are also fine mapped to the *Xa4* region where recombination

occurs frequently. Thus, it seems that a high frequency of recombination may partly be the cause for the generation of new resistance genes leading to a diversity of disease resistance genes in this genomic region. In addition, the diversity of *R* genes in the region containing *Xa4* may also be generated by unequal recombination and mispairing between duplicated sequences (Hulbert and Bennetzen 1991; Sudupak et al. 1993).

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