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Construction of a BAC contig containing the *xa5* locus in rice

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Abstract The recessive gene *xa5* confers resistance to bacterial blight in rice. To generate a physical map of the *xa5* locus, three RFLP markers RG556, RG207 and RZ390, closely linked to *xa5*, were used to screen a rice bacterial artificial chromosome (BAC) library. The identified overlapping BAC clones formed two small contigs which were extended to both sides by chromosome walking. The final physical map consisted of 14 BAC clones and covered 550 kb. Genetic analysis with an F_2 population showed that two RFLP markers 28N22R and 40F20R, derived from the BAC clones in the contig, flanked the *xa5* locus. To further delimit the location of the *xa5* locus, RFLP markers RG556 and RG207 were converted to sequence tagged sites and used to perform genetic analysis. The results indicated that the *xa5* locus was most likely located between RG207 and RG556. Among the BAC clones in the contig, one clone, 44B4, hybridized to both RG207 and RG556. This suggests that BAC clone 44B4 carried the *xa5* locus.

Key words Bacterial blight · Disease resistance · Physical map · *Oryza sativa*

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Introduction

Bacterial blight is one of the most serious rice diseases in irrigated and rainfed lowland ecologies throughout Asia. It is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The most effective method to control the disease is to develop host resistance. Currently, at least 19 genes conferring host resistance against the pathogen have been identified (Kinoshita 1995). Several bacterial blight resistance genes, mapped to different chromosomes, are based on morphological and/or molecular markers. *Xa1* and *Xa2* were mapped on the short arm of chromosome 4 (Yoshimura et al. 1992) and *Xa3*, *Xa4*, *Xa10* and *Xa21* were mapped on the short arm of chromosome 11 (Yoshimura et al. 1995; Ronald et al. 1992). The *xa5* gene is located on chromosome 5 (Yoshimura et al. 1985) and *xa13* maps to chromosome 8 (Zhang et al. 1996).

The mapping of these resistance genes permits marker-assisted selection in rice crop-improvement programs. Pyramiding of two, three and four genes for bacterial blight resistance is now possible (Huang et al. 1997). The pyramid lines show a higher and wider spectrum of resistance. The mapping of resistance genes also facilitates the generation of a physical map and, ultimately, the cloning of resistance genes. Song et al. (1995) cloned *Xa21* through a map-based cloning procedure. Yoshimura et al. (1996) identified a single YAC clone containing *Xa1*. The cloning of these genes will facilitate the study of the molecular mechanisms of resistance to bacterial blight. Both *Xa21* and *Xa1* are dominant; thus the results from the study of *Xa21* and *Xa1* would, most likely, not be applicable to recessive genes such as *xa5* and *xa13*.

The recessive gene *xa5* confers resistance to a broad spectrum of *Xoo* in the Philippines. It was first identified by Petpisit et al. (1977) and mapped by Yoshimura et al. (1995) using a set of NIL and RFLP markers. In the population they employed, no recombinant was

observed between *xa5* and the three RFLP markers, RZ390, RG556 and RG207. Recently, Blair and McCouch (1997) fine-mapped the *xa5* region and suggested that it is located between RG556 and RZ390.

During the course of our physical mapping of the rice genome and from the physical map obtained we developed target-specific DNA markers for marker-assisted rice breeding, and generated overlapping BAC clones near the region of *xa5*. This mapping study indicates that the physical map encompasses the *xa5* locus.

Materials and methods

Mapping population

New Plant Type line IR65598-112-2 (susceptible) was crossed to IRBB5 (resistant) which contains *xa5*. The F_1 was selfed to obtain an F_2 population. The F_2 mapping population consists of 256 individuals which were planted in the greenhouse for disease-reaction evaluation and to obtain tissue for DNA isolation.

Phenotype scoring

F_2 plants were inoculated with race 2 (PXO86) of *Xoo* using the leaf clipping method of Kauffman et al. (1973). Reaction to the pathogen was determined 18 days after inoculation. Plants were scored resistant or susceptible based on the lesion lengths compared to their parents.

DNA isolation and Southern-blotting analysis

Genomic DNA was extracted from a 4-week-old leaf using the method of Dellaporta et al. (1983). Southern-blotting analysis followed the standard procedure (Sambrook et al. 1989). RFLP marker probes were kindly provided by Susan McCouch (Cornell University). Additional probes for chromosome walking were generated by TAIL-PCR of BAC clones (Liu and Whittier 1995). Polymorphic markers were scored and analyzed with MAPMAKER 3.0. (Lander 1993).

Amplicon length-polymorphism (ALP) analysis

Amplicon length polymorphism was used to analyze the segregation of DNA markers and *xa5*. The first set of primers (Huang et al. 1997) is from RFLP marker RG556 (Causse et al. 1994). ALP was detected when the PCR products were digested with the restriction enzyme *Mae*II. The other set of primers, derived from RG207, were kindly provided by John Bennett. The sequence of RG207F is 5′-ATT GCC TAC GAC GAA GAT AGC-3′ while RG207R is 5′-GCC ATG GCG ACT GTC AGT CG-3′. Polymorphism was detected with primers from RG207 without restriction digestion. PCR analysis followed the procedure described by Hittalmani et al. (1995).

Preparation of a high-density BAC filter

A bacterial artificial chromosome (BAC) library of IR64 was used for this study (Yang et al. 1997). The BAC library consists of 18 432 BAC clones in 48 384-well microtiter plates. High-density BAC filters (1536 BAC clones/filter) consisting of 12 filters per set were prepared as described by Yang et al. (1997).

Preparation of DNA from BAC clones

The alkali lysis procedure for plasmid isolation (Sambrook et al. 1989) was used to obtain BAC DNA with a few minor modifications. After the *Escherichia coli* cells were suspended, the same volume of lysis buffer (solution II) and neutralization buffer (solution III) was added sequentially. After centrifugation, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant to remove contaminating protein and then a 0.7-fold vol of isopropanol was added to precipitate DNA at room temperature for 30 min. The procedure of recovering DNA and removing RNA was as described by Sambrook et al. (1989).

Preparation of the ends of BAC clones by TAIL-PCR of BAC clones

The end fragments of BAC clones were obtained by TAIL-PCR as described in Liu and Whittier (1995). A set of TAIL-PCR primers was synthesized according to the sequence of vector pBeolBAC 11 (Shizuya et al. 1992), and the primer sequences were

BF1 5′-GACGTTGTAAAACGACGGCCAGT-3′
BF2 5′-GTAATACGACCACTATAGGGCGA-3′
BF3 5′-GAGTCGACTCTGCAGGCATGCA-3′
BR1 5′-CTTCCGGCTCGTATGTTGTGTG-3′
BR2 5′-GAGCGGATAACAATTTCACACAGGA-3′
BR3 5′-AGGTGACACTATAGAATACTCA-3′.

Three arbitrary degenerate primers (AD1–AD3) were employed. The sequences of the AD primers are according to Liu and Whittier (1995) and Liu et al. (1995).

Colony hybridization and DNA fingerprinting

High-density filters were pre-hybridized in phosphate hybridization solution [0.5 M Na_3PO_4, (pH 7.2), 1 mM EDTA, 7% SDS, 100 μg/ml denatured sheared salmon sperm DNA] at 65°C for 4–6 h. Three kinds of probes, RFLP markers, entire BAC clone DNA and TAIL-PCR products, were employed. When the entire BAC clone DNA was used as a probe, the BAC DNA was digested with *Hin*dIII before labeling. For the RFLP markers, M13 primers were employed to amplify the RFLP markers as probes. TAIL-PCR products were used directly for labeling. All probes were labeled by the random primer kit (Rediprimer, Amersham, USA) with ^{32}P-dCTP (Amersham, USA). The labeled probe was added to pre-hybridization solution and hybridized at 65°C overnight. The filters were washed with 0.2 M phosphate buffer containing 0.1% SDS at 65°C twice for 20 min each with gentle shaking. For DNA fingerprinting with *Hin*dIII digestion, the washing condition was as described by Sambrook et al. (1989). The filters were exposed to X-ray film for 4–24 h depending on the signal intensity.

Determination of the insert size of BAC clones

The insert size of each BAC clone was determined by PFGE. The conditions for PFGE were switch time ramping from 5 to 10 s, temperature 11°C, 9 V/cm, pulse angle 110° using 0.5 × TAE buffer for 4 h. The size of each BAC clone was estimated based on its migration as compared to lambda size-markers.

Results

Identification of overlapping BAC clones near the *xa5* locus

A bacterial artificial chromosome (BAC) library of IR64 (Yang et al. 1997) was used to identify overlapping BAC clones near *xa5*. Three RFLP markers, RZ390, RG556 and RG207, closely linked to *xa-5* (Yoshimura et al. 1995; Blair and McCouch 1997), were used to screen the BAC library. Two overlapping BAC clones, 28N22 and 9E8, were obtained by RZ390 (Fig. 1). Two other BAC clones, 42J19 and 44B4, were identified by RG556 (Fig. 2 A) and four BAC clones, 44B4, 7J14, 39N11 and 41N1, were selected by RG207 (Fig. 2 B). Both RG556 and RG207 cross-hybridized to one clone, 44B4, with an insert size of 136 kb, integrating the five BAC clones into a single contig (Fig. 2 A and B). The screening with the three RFLP markers RZ390, RG556 and RG207 resulted in two contigs (Figs. 1 and 2). To integrate the two contigs, a clone, 42J19, located at the end of one contig was chosen as a probe to hybridize with contig RZ390 (Fig. 3). The BAC clone 42J19 overlapped with 44B4 at one end (Fig. 3 and Fig. 2 A) and overlapped with 9E8 in another end (Fig. 3) integrating the two contigs into one (Fig. 4). To extend the contig from both sides, two clones from both ends of the contig, 28N22 and 7J14, were used as probes to screen the BAC library. Three clones, 25P14, 8L9 and 46D22, were identified by 28N22 while four clones, 28P14, 40F20, 4MI and 18N18, were obtained with 7J14. The final contig consisted of 14 BAC clones spanning 550 kb (Fig. 4). The physical distance between RG207 and RZ390 is 220 kb. The order of the three RFLP markers on the physical map is RZ390, RG556 and RG207, which is the same as that determined based on genetic analysis by Blair and McCough (1997).

Fig. 1 DNA fingerprinting of two BAC clones, 9E8 and 28N22. BAC clone DNA from 9E8 and 28N22 were digested with *Hin*dIII and the resulting blot was probed with labeled 28N22

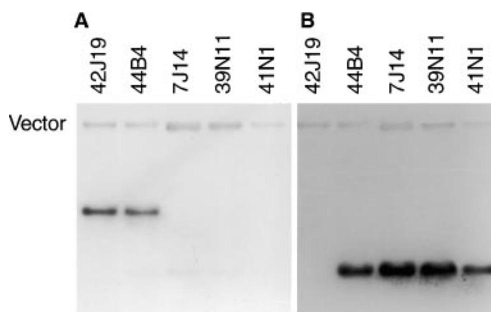

Fig. 2 Identification of overlapping BAC clones with RFLP markers RG556 (**A**) and RG207 (**B**). DNA isolated from five BAC clones, 42J19, 44B4, 7J14, 39N11 and 41NI, were digested with *Hin*dIII and the resultant blot was first probed with RFLP marker RG556, (**A**) and then with RFLP marker RG207 (**B**)

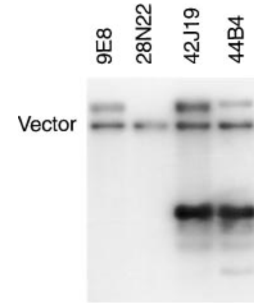

Fig. 3 DNA/DNA hybridization analysis. BAC clone DNA from 9E8, 28N22, 42J19 and 44B4 were digested with *Hin*dIII and the resultant blot was probed with labeled 42J19

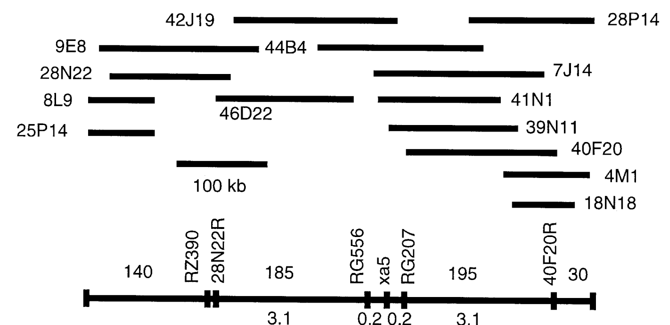

Fig. 4 Alignment of genetic and physical maps of *xa5* locus. The physical map is shown as overlapping BAC clones. Each BAC clone was represented by a *solid line* with line length equal to the cloned DNA fragment size estimated by PFGE. The genetic map, based on multiple point analysis, is shown with the genetic distance (cM) below the map. The *numbers* above the genetic map are for the physical distance (kb)

The BAC contig encompasses the *xa-5* locus

No recombinants between the three RFLP markers RZ390, RG556, RG207 and *xa5* were observed in previous studies (Yoshimura et al. 1995; Blair and McCouch 1997). On the one hand, this indicates that the *xa5* locus is located close to the three RFLP markers. On the other hand, it did not tell us if the RFLP markers flank *xa5*. To determine that the BAC contig contains the *xa5* locus, and to develop more DNA markers closely linked to *xa5* from the BAC

contig, an F_2 mapping population was analyzed with DNA markers isolated from BAC clones on the contig.

Ends of the BAC inserts were amplified with TAIL-PCR (Liu and Whittier 1995) and used as DNA probes. Out of the many PCR probes tested, two generated single-copy polymorphic bands. Other probes did not detect any polymorphisms or produce multiple bands that could be used for mapping analysis. The first polymorphic marker was derived from BAC clone 28N22 and named 28N22R. Linkage analysis indicated that 28N22R was about 3.1 cM from *xa-5* (Fig. 4). Another polymorphic marker was from the right end of 40F20. This marker, 40F20R, was placed on the other end of *xa5*. RFLP marker 40F20R was 5.5 cM from 28N22R and 3.1 cM from *xa5* (Fig. 4). Therefore, it was concluded that the BAC contig contained the *xa5* locus.

Identification of a BAC clone(s) carrying the *xa5* locus

In order to place the *xa5* locus within a specific BAC clone(s), the locus has to be mapped with high resolution. The RFLP markers were sequenced and primers were generated to re-examine their linkage to *xa5*. The PCR products generated from primer pairs derived from RG207 produced an ALP between IRBB5 and IR65598 (Fig. 5). The PCR products for RG556 produced polymorphism only when the PCR products were digested with the restriction enzyme *Mae*II (Fig. 5). PCR products from RG390 showed no polymorphism despite using more than 30 restriction enzymes. PCR analysis with primers from RG556 and RG207 were then employed to survey an F_2 population of 256 individuals. Similar to the results obtained by Yoshimura et al. (1995) and Blair and McCouch (1997), *xa5*, RG556 and RG207 were closely linked and paired; any two of the three loci were only 0.5 cM apart. Multiple-point linkage analysis with MAPMAKER placed the *xa5* locus between RG556 and RG207 (Fig. 4). Since both RG556 and RG207 hybridized to the BAC clone 44B4, it is very likely that 44B4 carries the *xa5* locus.

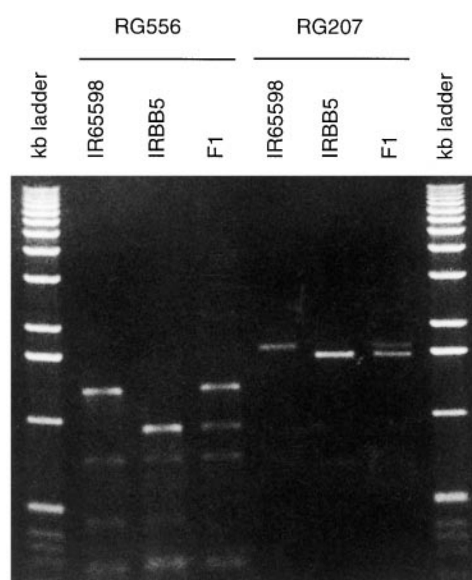

Fig. 5 Banding patterns of PCR products amplified from DNA of parents (New Plant Type line, IR65598 and IRBB5) and their F_1. STS primers were derived from the DNA sequence of RFLP markers RG556 and RG207. PCR products amplified with the RG556 primer were digested with *Mae*II before gel electrophoresis

Discussion

A contig map of the *xa5* locus was generated with 14 overlapping BAC clones. The fact that two contig-derived markers 28N22R and 40F20R flanked the *xa5* locus, provided evidence that the *xa5* locus was within the contig. From 28N22R to 40F20R, three BAC clones, 46D22, 44B4 and 40F20, formed a minimum path (Fig. 4). Thus, the *xa5* locus must be located within these three BAC clones (Fig. 4). The BAC clone, 44B4 contained both RG556 and RG207 marker loci and multipoint linkage analysis placed the *xa5* locus between them, indicating that the *xa5* locus was most likely within 44B4 (Fig. 4).

One interesting observation was the small ratio of physical distance to genetic distance. In the *xa5* region of chromosome 5 each cM is equivalent to about 61 kb. This number is much less than the 260 kb/cM of the overall estimate (Wu and Tanksley 1993), the 130 kb/cM of the *Xa1* region in chromosome 4 (Yoshimura et al. 1996), or the 141 kb/cM of the *Rf3* region in chromosome 1 (unpublished data). It remains to be seen if this is due to the population employed or due to the chromosome region studied.

Mapping of a locus to a physical map should facilitate the cloning of that locus. To clone the *xa5* locus, BAC clone 44B4 can be used as a starting point. The insert size of 44B4 is only 136 kb, from which a transcription map can be generated. BAC clone 44B4 can also be used as a probe to screen a library constructed with DNA isolated from a rice variety carrying the resistant *xa5* allele.

Construction of a contig map encompassing the *xa5* locus provides an opportunity to generate target-specific DNA markers from BAC clones. We generated two markers, 28N22R and 40F20R, from BAC clones. The advantage of generating markers from BAC contigs is that the markers are tightly linked to the target gene. Generating markers from BAC contigs is particularly useful when: (1) more RFLP markers are needed for a particular region; (2) existing RFLP markers around a target gene are not polymorphic, and (3) closer markers for accurate marker-assisted selection are required. The BAC-derived markers could be sequenced and converted to STS for application in crop improvement. As *xa5* is most likely within the BAC

clone 44B4, any markers derived from 44B4 would be tightly linked to *xa5* and hence suitable for marker-assisted transfer of the *xa5* gene in rice crop-improvement programs.

To increase the efficiency of progeny screening and future marker-assisted selection, three pairs of STS primers derived from three RFLP markers, RG556, RG207 and RZ390, were employed. The population was developed to transfer the *xa5* gene from IRBB5 to New Plant Type IR65598-112-2. STS primers were used to assist the gene transfer. The STS primer of RZ390 gave no polymorphic multiple bands while primers from RG556 and RG207 were used for ALP analysis. These two STS primers should be useful for other marker-assisted selection programs involving *xa5* (Huang et al. 1997).

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