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Chromosome landing at the bacterial blight resistance gene *Xa4* locus using a deep coverage rice BAC library

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Abstract *Xa4* is a dominantly inherited rice gene that confers resistance to Philippine race 1 of the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in rice. In order to isolate the gene by positional cloning, a bacterial artificial chromosome (BAC) library was constructed from genomic DNA isolated from an *Xa4*-harboring accession, IRBB56. The library contains 55,296 clones with an average insert size of 132 kb, providing 14 rice genome equivalents. Three DNA markers closely linked to *Xa4* were used to screen the library. The marker RS13, a resistance gene analogue that co-segregates with *Xa4*, identified 18 clones, of which four and six, respectively, were simultaneously detected by the other two markers, G181 and L1044. Fingerprinting and Southern analysis indicated that these clones overlapped and define an interval spanning 420 kb. In an F₂ population derived from an *indica* variety, IR24, and its *Xa4*-containing near isogenic line (NIL), IRBB4, the susceptible plants were screened in order to map the *Xa4* gene genetically and physically. Out of 24 insert ends isolated from the BACs in the contig, three revealed polymorphisms between IR24 and IRBB4. Two insert ends, 56M22F and 26D24R, flanked *Xa4*

and tomato (Kanazin et al. 1996; Leister et al. 1996, 1998, 1999; Yu et al. 1996; Aarts et al. 1998; Collins et al. 1998; Ohmori et al. 1998; Shen et al. 1998; Mago et al. 1999; Graham et al. 2000; Pan et al. 2000; Seah et al. 2000). Some of these RGAs have been shown to be associated with known resistance (*R*) gene loci in these plants. Thus, such RGAs can be exploited in the chromosome landing strategy for isolation of these *R* genes.

Bacterial blight (BB) is one of the most serious rice diseases in Asia. It is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). Currently more than 20 genes that confer

Host Resistance to the pathogen have been identified (for reviews, see Kinoshita 1995 and Gnanamanickam 1999).
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et al. 1999; Lin et al. 1996; Zhang et al. 1998). Genetic mapping of these resistance genes not only permits marker-assisted selection in rice breeding programs, but also facilitates cloning of these genes. So far, two BB resistance genes, *Xa21* and *Xa1*, have been cloned (Song et al. 1995; Yoshimura et al. 1998), and two recessive BB resistance genes, *xa5* and *xa13*, have been physically mapped (Yang et al. 1998; Sanchez et al. 1999). *Xa4* used to be the most widely exploited resistance gene in many Asian rice breeding programs and conferred durable resistance in many commercial rice cultivars (Mew et al. 1992; Li et al. 1999). It forms part of a resistance gene cluster including *Xa3*, *Pi-1(t)*, *Pi-k*, and *Pi-f* on chromosome 11 (Xiao et al. 1992; Leister et al. 1998, 1999). Moreover, the use of rice accessions in which *Xa4* is combined with other BB resistance genes could result in a higher resistance level and an even wider resistance spectrum than those associated with a single resistance gene (for review, see Huang et al. 1997; Zheng et al. 1998; Gnanamanickam et al. 1999). These studies indicate a new approach to the further utilization of BB resistance genes that have been widely exploited. Therefore, we have chosen *Xa4* as a target for positional cloning.

Xa4 was first localized by Yoshimura et al. (1992) on rice chromosome 11, distal to the RFLP marker G181. Then Li et al. (1999) mapped it between RZ536 and G2132b. Recently, several RGAs amplified from rice by using degenerate primers have been mapped in this region (Leister et al. 1998, 1999; Mago et al. 1999). In order to screen for RGAs associated with *Xa4* locus, we also exploited the PCR-based approaches. One of the RGAs, RS13, which has high sequence similarity to cloned NBS-LRR genes, had been mapped to the distal flank of G181 (Zheng et al., unpublished data). By using an F₂ population of 467 individuals derived from an *indica* variety, IR24, and its *Xa4* NIL, IRBB4, RS13 had been found to co-segregate with *Xa4* and was localized between the RFLP markers G181 and L1044 (Wang et al. 2000). Thus, RS13 could be used to land at the *Xa4* locus. In order to do so, a BAC library for IRBB56, a pyramid accession with three BB resistance genes including *Xa4* (Huang et al. 1997), was constructed. The markers RS13, G181 and L1044 were used to screen the library. Then, a contig was developed that spanned approximately 5 kb (IRBB56 contig) (Wang et al. 2000).

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Isolation of insert ends from BAC clones

The insert ends of BAC clones were obtained by TAIL-PCR as described by Liu and Whittier (1995). The primers for TAIL-PCR were synthesized as described by Yang et al. (1998).

DNA isolation and Southern analysis

Total DNA was extracted from individuals that were absolutely susceptible to *Xoo* using the method of McCouch et al. (1988). Southern analysis followed the standard procedure (Sambrook et al. 1989). RFLP markers G181 and L1044 were kindly provided by the Rice Genome Project of Japan (Harushima et al. 1998).

Results

BAC library construction and characterization

A rice BAC library, consisting of 55,296 clones, was constructed from the rice accession IRBB56. Analysis of a random sample of 522 BACs picked from the library during the course of library construction showed an average insert size of 132 kb, with a range of 30–260 kb. The 522 BACs were grouped by insert size and the insert size of each clone was plotted against the frequency of each group of clones represented in the library (Fig. 1). Based on this analysis, over 80% of the insert-containing clones were estimated to have an average insert size greater than 100 kb. Of the clones larger than 100 kb, 60% were between 140–170 kb and more than 85% had inserts greater than 130 kb. Considering the average insert size and a haploid genome size of 430 Mb (Arumuganthan and Earle 1991), the coverage of the library was about 14 genome equivalents, resulting in a 99.99% probability of recovering any specific sequence

of interest, though some 17% of clones did not contain inserts. To determine the representation of organelle DNAs in the library, the library were screened with three different chloroplast genes spaced equidistantly around the 133 kb barley chloroplast genome and four rice mitochondrial genes, respectively. Results from this screen showed that approximately 0.85% of library sequences were chloroplast DNA and 0.11% were mitochondrial DNA homologs (data not shown).

BAC library screening with markers linked to *Xa4*

The three DNA markers G181, RS13, and L1044, which are linked to *Xa4*, were used to screen the library, resulting in 13, 18, and 106 hits, respectively (data not shown). The high hit number detected by L1044 implied that L1044 might contain multicopy sequences. The marker RS13, a resistance gene analogue that co-segregates with *Xa4*, picked out 18 candidate BAC clones, of which 1F21, 26D24, 56M22, and 111E1 were simultaneously detected by the RFLP marker G181; and 1F21, 33M8, 56M22, 61A13, 104B15, and 111E1 were simultaneously detected by L1044 (Table 1). These results implied that these clones may overlap and cover the region between L1044 and G181 on chromosome 11.

Identification of candidate BACs at the *Xa4* locus

It is difficult to handle all of the BACs picked out by the three probes linked to *Xa4*. So, we focused on the 18 clones recognized by RS13, some of which were simultaneously detected by G181 and L1044. The insert sizes

Fig. 1 Insert size distribution of BAC clones in the IRBB56 BAC library. BAC DNAs of 522 randomly selected clones were digested with *NotI*. The insert sizes were determined by CHEF electrophoresis and plotted against frequency of occurrence

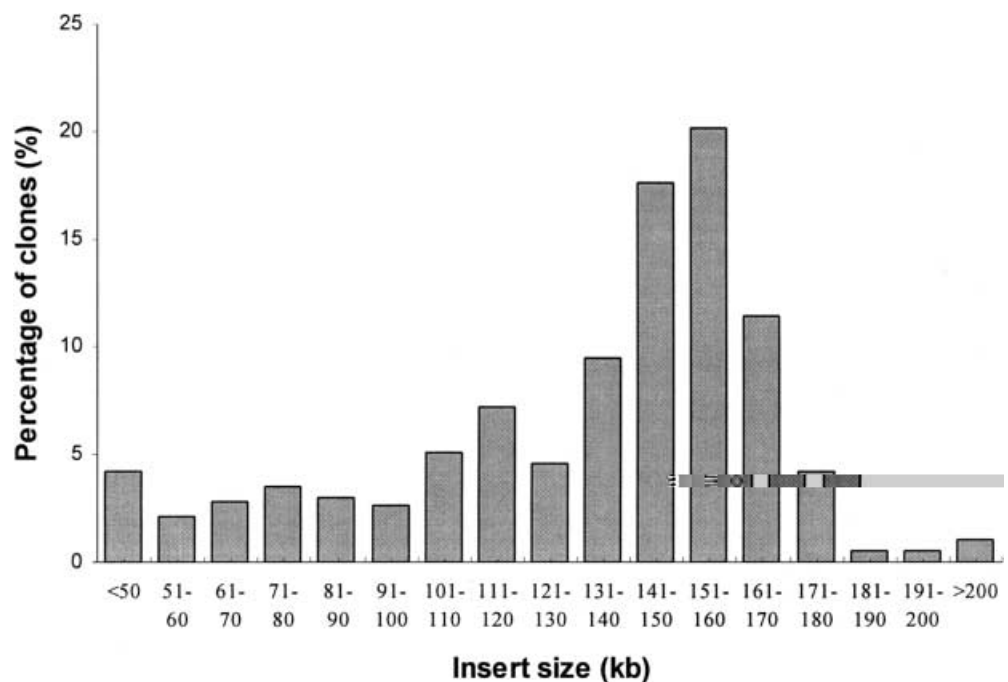


Table 1 Candidate BAC clones covering the *Xa4* locus

Clone no. ^a	Clone address	Insert size (kb) ^b	Positive probe
1	56M22	140	RS13, L1044, G181
2	111E1	140	RS13, L1044, G181
3	117I22	175	RS13
4	106P13	180	RS13
5	50B1	150	RS13
6	43B6	160	RS13
7	29H13	170	RS13
8	26D24	160	RS13, G181
9	45B20	145	RS13
10	104B15	180	RS13, L1044
11	61A13	140	RS13, L1044
12	33M8	165	RS13, L1044
13	1F21	130	RS13, L1044, G181
14	37E12	170	RS13
15	81P3	150	RS13
16	95F11	160	RS13
17	99P13	160	RS13
19	110C24	155	RS13

^aThe first 12 BACs was used to construct a contig covering the *Xa4* locus

^bThe insert size was determined by CHEF electrophoresis after *NotI* digestion

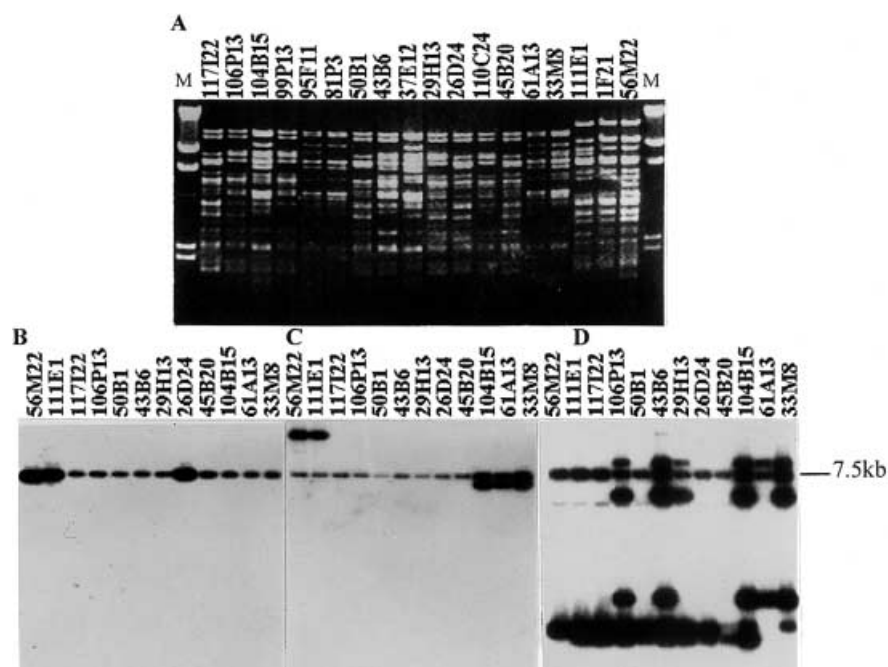
in these clones were in a range of 130–180 kb, as determined by PFGE analysis of *NotI*-digested BACs (Table 1). The *HindIII* restriction patterns revealed that six BAC clones (1F21, 37E12, 81P3, 95F11, 99P13, and 110C24, as shown in Fig. 2A) were completely included by others, and therefore these were not used in our further investigation. The other 12 BACs were subjected to Southern analysis to confirm their identity. In *HindIII* digests of individual BACs, G181, L1044 and RS13

probes detected 1–5 hybridizing bands, and thus, all were indeed positive for all three probes. The hybridizing bands also revealed the locations of the three probes in the positive clones. G181 detected one band common to 56M22, 111E1 and 26D24 (Fig. 2B). Thus, G181 was located in the overlap between these clones. L1044 detected bands at two different positions: one band was common to 56M22 and 111E1, both harboring G181, the other was common to 104B15, 33M8, and 61A13 (Fig. 2C), implying that homologs of L1044 were situated in different regions. Nevertheless, according to the genetic map (Wang et al. 2000), L1044 should be located in the overlap between 104B15, 33M8, and 61A13 (see below). When RS13 was used as a probe, two to five hybridizing bands were seen in the *HindIII* digests of the BACs, and each band was common to at least two BACs (Fig. 2D), confirming that these BACs overlap and implying that a cluster of RS13 homologs exists in the region. In a rice accession harboring *Xa4*, RS13 could detect up to five *HindIII* bands (data not shown). It is noted that among the BACs recognized by RS13, the clones 106P13 and 43B6 included all the hybridizing bands detectable (Fig. 2D).

Construction of a BAC contig in the *Xa4* region

The 12 overlapping BACs were arranged to form a contig based on their *HindIII* and *NotI* restriction patterns. To confirm this arrangement and estimate the length of the contig, Southern analyses were conducted by using forward and reverse insert ends from the BACs as probes (Fig. 3). As the reverse insert end of 56M22 (56M22R) detected three unique extra bands in 56M22 (Fig. 3A), 56M22R should represent one of the outermost ends of the contig. The forward insert end of

Fig. 2A–D Analysis of candidate clones covering the *Xa4* locus. **A** *HindIII* restriction patterns of 18 candidate BACs. M, marker (*HindIII* digest of λ DNA). **B–D** Hybridization patterns revealed by the probes G181 (**B**), L1044 (**C**) and RS13 (**D**)



56M22 (56M22F) detected two bands in 56M22 that were shared by 111E1, 117I22, 106P13, 50B1, 29H13, 26D24 and 45B20 (Fig. 3B); therefore, the insert end 56M22F overlapped with these BACs. Similarly, it was deduced that the forward insert end of 26D24 (26D24F) overlapped with 56M22 and 111E1 (Fig. 3C). The bands situated at both the reverse insert end of 26D24 (26D24R) and the reverse insert end of 43B6 (43B6R) were shared by 117I22, 106P13, 50B1, 43B6, 29H13, 26D24 and 45B20 (Fig. 3D and E); thus, 26D24R and 43B6R overlapped with these BACs. The forward insert end of 43B6 (43B6F) overlapped with 104B15, 61A13 and 33M8 (Fig. 3F). The forward insert end of 104B15 (104B15F) overlapped with 117I22, 106P13, 50B1, 43B6, 29H13, 45B20 and 33M8 (Fig. 3G). The reverse end of 104B15 (104B15R) detected two bands shared by 104B15 and 61A13 (Fig. 3H), indicating that their ends were probably situated at approximately the same site forming the other end of the contig. This deduction was then confirmed by probing *Hind*III-digested BACs in the contig with the insert ends of 61A13 (data not shown). The insert of 61A13 was shorter than that of 104B15, as shown by their *Hind*III-restriction patterns (Fig. 2A) and insert size determination based on PFGE analysis after *Not*I digestion (Table 1). Thus, 56M22 and 104B15 represent the two outermost clones on each end of the contig, and are 140 kb and 180 kb in length, respectively. Because both 56M22F and 104B15F overlap with 117I22 (Fig. 3B and G), which is 175 kb in length and shares 55 kb with 56M22 and 20 kb with 104B15, as shown by their *Hind*III and *Not*I restriction patterns (Fig. 2A, data not shown), the physical distance between 56M22 and 104B15 is 100 kb. Thus, the ordered contig spans about 420 kb. This contig is covered by three BACs, namely, 56M22, any one of the

five central BACs (117I22, 50B1, 29H13, 45B20, and 106P13), and 104B15.

Identification of BACs contain the *Xa4* allele

In order to locate the *Xa4* allele in the BAC contig, an F₂ population containing 1401 plants was obtained from a cross between IR24 and its *Xa4* NIL IRBB4, in which the resistant gene *Xa4* was completely dominant to its susceptible allele. It was therefore fairly easy to score the susceptible individuals. The ratio between the resistant and susceptible individuals was consistent with the expected single-gene segregation ratio of 3:1 (339 susceptible out of 1401 plants, $\chi^2=0.482$, $P=0.4-0.5$). However, the resistant phenotype might be attributable to either the resistant genotype or to the failure of *Xoo* infection. In addition, both homozygous and heterozygous individuals with *Xa4* could contribute to resistant phenotypes, so that the exchange events between the linked markers and *Xa4* might not be revealed. Therefore, only the 339 susceptible individuals were selected for linkage analyses. The NIL parents were probed with 24 isolated BAC insert-ends by Southern analysis. Three of them revealed polymorphisms between the NIL parents; for these, 56M22F detected three recombinants, 26D24R five recombinants, and 104B15R six recombinants among the susceptible individuals. Since the recombinants detected by 56M22F were different from those detected by 26D24R and 104B15R, *Xa4* should map in the region between 56M22F and 26D24R, an interval corresponding to a genetic distance of 1.1 cM and a physical length of approximately 90 kb. Based on the physical relationship between the BACs, the *Xa4* allele was located in the middle six clones of the contig as shown in Fig. 4B.

Fig. 3A–H Southern hybridization of 12 overlapping BACs digested with *Hind*III and probed with the insert-ends from some overlapping BACs. The insert-ends used as probes are indicated above the photographs. The arrows indicate the end fragments and their counterparts in the overlapping clones

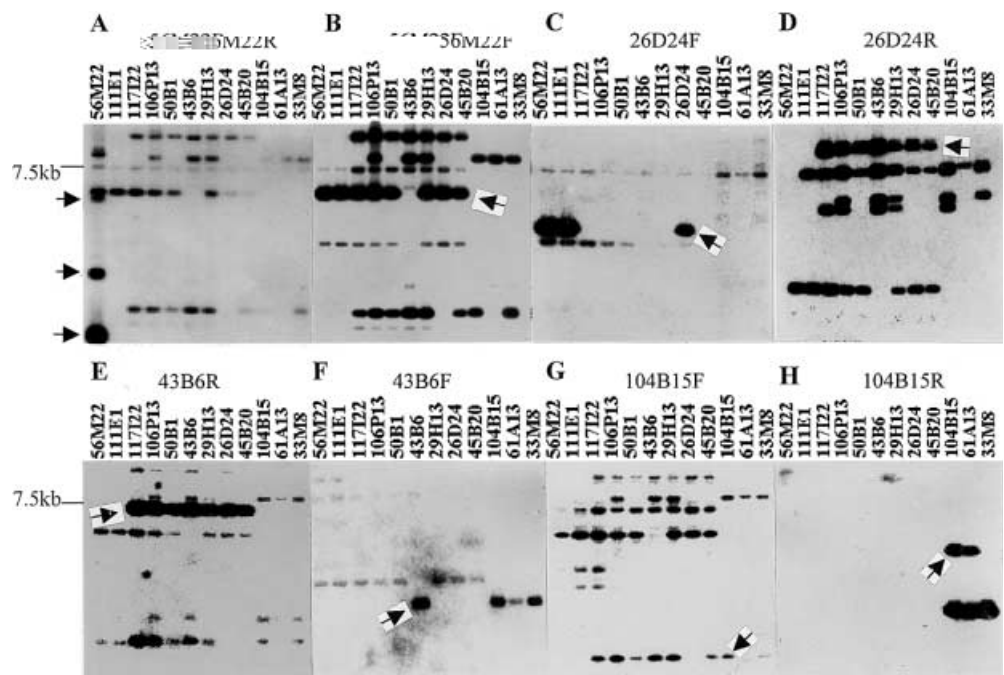
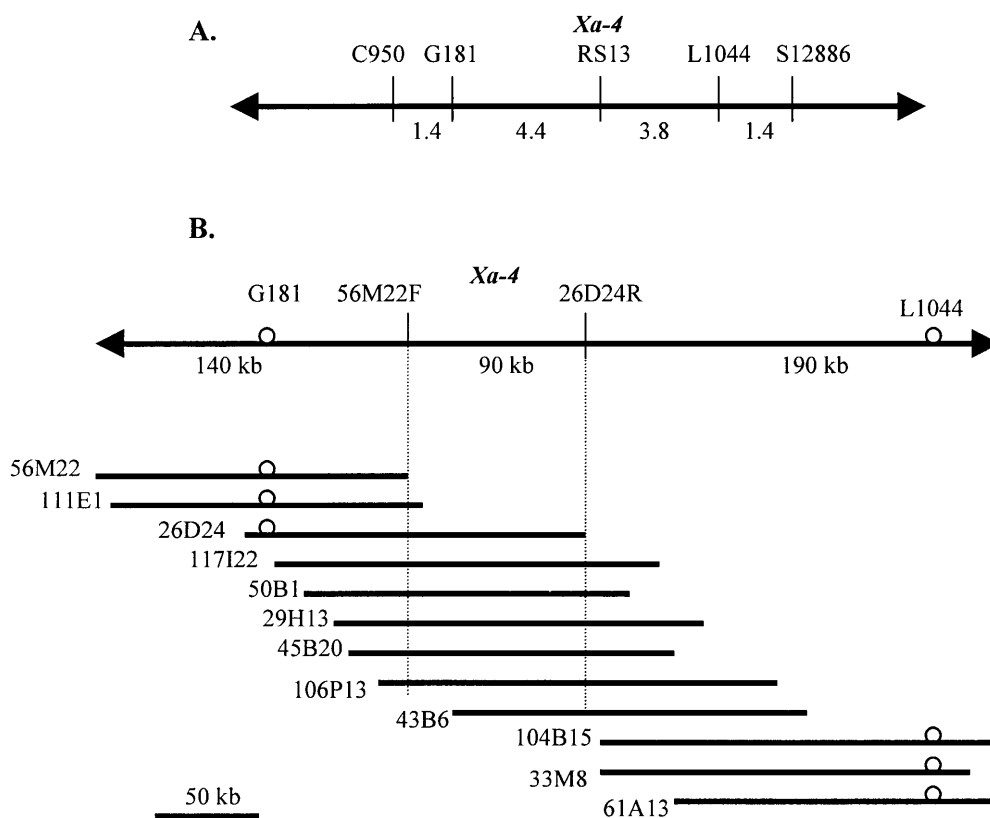


Fig. 4A, B Genetic and physical maps of the *Xa4* locus.

A The *double arrowed line* represents chromosome 11. The *numbers under the line* are map distances in Centimorgans.

B The *doubly arrowed line* represents the chromosome region surrounding the *Xa4* locus, covered by the three cloned DNA fragments, whose sizes were estimated by CHEF electrophoresis. The *horizontal lines below the map* represent the BACs named on the *left*. The region between the dotted vertical lines probably contains the *Xa4* locus



Discussion

Currently, quite a few BAC libraries for various rice varieties have been constructed by different laboratories (Tao et al. 1994; Wang et al. 1995; Zhang et al. 1996; Nakamura et al. 1997; Yang et al. 1997). However, the usefulness of these libraries is limited because their size is relatively small (3–8 genome equivalents), or they do not contain some specific target genes. In the present study, in order to isolate the *Xa4* gene, a BAC library, characterized by high clone number, large insert fragments, deep coverage of the rice genome, and a low level of organellar DNA, was constructed from the rice accession IRBB56. IRBB56 was chosen for BAC library construction because it carries not only the *Xa4* gene, but also *xa5* and *xa13*, two recessive genes conferring resistance to *Xoo* (Huang et al. 1997). In addition, IRBB56 had been developed from IR24, a restorer line that has been widely exploited in hybrid rice production. Thus, the library could be used, too, for screening for the restorer gene for cytoplasmic male sterility and other important genes. The library size of fourteen genome equivalents makes it one of the most comprehensive BAC libraries available in rice and provides a 99.99% probability that any desired rice sequence is represented in the library.

The deep coverage of the BAC library was confirmed by the high hit numbers for the three markers (G181, RS13, and L1044) linked to the *Xa4* gene. The hit

numbers for G181 and RS13 were in the range expected based on the library coverage. However, the hit number detected by L1044 was unexpectedly high. Southern analysis of the *Hind*III-digested BACs indicated that G181 was a single-copy probe and RS13 was a clustered multicopy probe (Fig. 2B and D). Hybridization patterns shown in Fig. 2C clearly illustrated that there were two copies of L1044 located in each of the five positive BACs, and analysis of the other BACs that were positive with L1044 revealed that there were other copies further away (data not shown). Thus, L1044 must be a dispersed multicopy probe, which would account for its high hit number. It was also comprehensible that three BACs, 56M22, 111E1, and 1F21, did not cover the whole contig though they were simultaneously detected by all the three markers.

By making use of conserved domains of cloned *R* genes, investigators have obtained RGAs associated with known *R* gene loci (Leister et al. 1996; Mago et al. 1999; Seah et al. 2000). These RGAs can be used as a starting point for the isolation of *R* genes following the chromosome landing strategy. Previously, we had mapped the RGA fragment RS13 between L1044 and G181, cosegregating with the *Xa4* (Wang et al. 2000). In the present study, based on the construction of a deep-coverage BAC library and the development of an F_2 population containing 1401 plants from a cross between IR24 and its *Xa4* NIL IRBB4, we constructed a contig spanning approximately 420 kb with three BACs as the minimum tiling. Then the *Xa4* allele was localized

between two insert ends, 56M22F and 26D24R, on the basis of three and five recombinants in 339 susceptible individuals, respectively. The physical and genetic distances between the two insert ends were approximately 90 kb and 1.1 cM, respectively. The ratio of physical to genetic distance (< 100 kb/cM) was much smaller than the average for rice (300 kb/cM) (Arumuganathan and Earle 1991; Harushima et al. 1998). The higher recombination rate in this region, which greatly facilitated our fine-scale mapping of the *Xa4* locus, may be attributable to its location close to the telomere on the longer arm of chromosome 11.

Other studies on the cloning and mapping of dominant resistance genes have revealed that in most cases the situations were more complicated than initially anticipated. Disease resistance genes frequently occur in tightly linked clusters. Analysis of the *Xa21* gene family in rice revealed that at least seven family members are located within a 230-kb genomic region. Apart from *Xa21* itself, *Xa21D* displayed a resistance spectrum identical to that observed for *Xa21* but conferred only partial resistance to *Xoo* (Song et al. 1997; Wang et al. 1998). It has been shown that there are several resistance genes, including two BB resistance genes (*Xa4* and *Xa3*) and three rice blast disease resistance genes (*Pi-1(t)*, *Pi-f*, and *Pi-k*), located in the region distal to G181 on chromosome 11 (Xiao et al. 1992; Leister et al. 1998, 1999). It is also noteworthy that quite a few RGAs have been located in this region (Leister et al. 1998, 1999; Mago et al. 1999; Zheng et al., unpublished data). RS13 has a sequence that is highly similar to genes of the NBS-LRR resistance gene class. In a previous study, it was used to probe varieties harboring the *Xa4* gene, and five *Hind*III bands were detected. In the present study, a BAC contig was derived from a BAC library for IRBB56, an *Xa4*-bearing line. When RS13 was used as a hybridization probe for *Hind*III digests of the BAC clones, two BAC clones (43B6 and 106P13) located in the middle of the contig, were found to include all of the five bands detectable with the probe. The multiple bands detected by RS13 imply that a family of resistance gene homologs exists at the *Xa4* locus, and complex rearrangements may have shaped this region. Recently, Li et al. (1999) identified an allele, referred to as the “defeated” resistance gene *Xa4^T*, at the *Xa4* locus in the *indica* cultivar Teqing, that also provides proof that a cluster of genes of the *Xa4* family is located in this region. Although the *Xa4* locus has thus far been considered to consist of a single dominant resistance gene, it is quite possible that the locus contains a cluster of closely linked resistance gene homologs. The BAC clone 106P13, which includes all five *Hind*III bands detectable by RS13 and covers the region between 56M22F and 26D24R, thus presumably carries all members of the *Xa4* gene family and is suitable for the isolation of the *Xa4* gene.

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