Localizing the Bacterial Blight Resistance Gene, *Xa22(t)*, to a 100-Kilobase Bacterial Artificial Chromosome

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ABSTRACT

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The rice bacterial blight resistance gene, *Xa22(t)*, provides resistance to a broad spectrum of *Xanthomonas oryzae* pv. *oryzae* isolates. Here, we localized the gene to a small 100-kb fragment of chromosome 11 by a combination of genetic recombination analysis and physical mapping. Mapping was done with two F_2 populations from the cross between Zhachanglong and Zhenzhuai. The first population consisted of 248 random individuals and 404 highly susceptible individuals selected from an F_2 population of more than 2,000 individuals and was used to construct a linkage map around the *Xa22(t)* locus. For the second F_2 population, 7,680 plants were examined with simple sequence repeat markers

flanking the *Xa22(t)* locus to identify recombinants useful for fine-genetic mapping. Two large-insert bacterial artificial chromosome (BAC) libraries (from cvs. Teqing and Minghui63) were screened with a marker (R1506) which cosegregated perfectly with *Xa22(t)* in the first population. Restriction mapping of the resulting BAC clones enabled a physical map of the area to be constructed, and subclones from the BAC clones provided additional restriction fragment length polymorphism probes which could be placed on the fine-structure genetic map using the recombinants from the second mapping population. The *Xa22(t)* locus was mapped to a $\approx$ 100-kb interval delimited by the R1506 marker and a subclone from the M3H8 BAC clone.

Additional keywords: genetic map, physical map, rice, *Xanthomonas oryzae* pv. *oryzae*.

Bacterial blight seriously reduces rice production worldwide (16). More than 20 bacterial blight resistance genes have been identified and mapped on rice chromosomes (5). Two of them, *Xa21* (21) and *Xa1* (32), have been cloned successfully by a map-based cloning approach. Three additional genes have been physically mapped: two recessive bacterial blight resistance genes, *xa5* and *xa13* (20,29), and one widely used dominant gene, *Xa4* (22,26).

Xa22(t) was identified from a landrace (Zhachanglong [ZCL]) of the Yunnan province in southwest China, a center of diversity for Asian cultivated rice (*Oryza sativa* L.). *Xa22(t)* showed high levels of resistance to 16 of the 17 bacterial blight strains tested and was mapped on the long arm of chromosome 11 (12). It was reported on the short arm in that article. It should be on the long arm according to new molecular map of Harushima et al. (7). For rice, a cereal model plant, high-density genetic linkage maps (2,7) are available, including simple sequence repeat (SSR) markers (23), and its sequence frameworks have been gained by several groups, including a group in China (33), Monsanto, Syngenta (6), and an international consortium. Map-based cloning is now an efficient approach to isolate functional genes if they can be accurately mapped. Four rice disease-resistant genes, *Xa21* and *Xa1* (conferring resistance to bacterial blight) and *Pib* and *Pi-ta* (providing resistance to blast, *Magnaporthe grisea*), were identified by map-based cloning strategy (1,21,27,32).

The present study constructs a fine map of the target gene, *Xa22(t)*. (i) Tightly linked markers were identified on both sides by using F_2 analysis. (ii) Bacterial artificial chromosome (BAC) clones were isolated by screening with the tightly linked markers and were identified by Southern hybridization. The overlapping

BAC clones made up contigs that were extended by chromosome walking. (iii) Recombinant plants, which showed unilateral cross-overs on either side of the target gene, were identified by markers and subclones. The target region was delimited to one BAC clone.

MATERIALS AND METHODS

Pathogen strains and disease evaluation. PXO61 (the Philippine *Xanthomonas oryzae* pv. *oryzae* race 1, provided by T. W. Mew) was used to assay resistance in the two mapping populations. The bacterial strains were seeded on a potato semisynthetic agar medium (24) and incubated at 28°C for 3 days. Inoculum was prepared by suspending the bacterial mass with sterilized water at a concentration of approximately 6×10^8 cells per ml, measured by the barium sulfate turbidimetry method.

Rice seedlings were transplanted to the disease nursery 30 days after sowing. The space between plants in a row was 12 cm, and the rows were 24 cm apart. At booting stage ($\approx$ 40 days after transplanting), five to seven of the uppermost fully expanded leaves of each plant were inoculated by the leaf clipping method, in which the leaf was cut with a pair of scissors dipped in the bacterial suspension (11). For disease scoring, the longest lesion for each of two or three undamaged leaves per plant was measured 20 days after inoculation. A plant was classified as resistant if the average lesion length was $\leq$ 9.0 cm, and susceptible if the lesion length was $>$ 9.0 cm.

Populations for inheritance study. Two F_2 populations derived from the cross between ZCL (resistant parent carrying *Xa22(t)*, *Oryza sativa* subsp. *japonica*) and Zhenzhuai (ZZA; susceptible to all *X. oryzae* pv. *oryzae* isolates, *O. sativa* subsp. *indica*) were constructed. The plants of two F_2 populations were inoculated with *X. oryzae* pv. *oryzae* pathogen strain PXO61 at booting stage. A sample of 248 random plants and a sample of 404 susceptible plants were taken from the first F_2 population, which consisted of over 2,000 plants. The random sample was used to

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confirm the resistance from cv. ZCL to *X. oryzae* pv. *oryzae* PXO61 controlled by only one gene and to construct a linkage map flanking the *Xa22(t)* locus. The susceptible sample was used to identify additional recombination events between the markers and *Xa22(t)*. The F_3 families were derived from the 248 random plants and 404 susceptible plants for confirming the reaction of F_2 plants to PXO61. The second F_2 population of 7,680 plants was examined using SSR markers RM144 and RM224 that flanked the *Xa22(t)* locus for identifying individuals derived from recombination events between markers and *Xa22(t)*. These populations and their parents were cultivated in a test field at Huazhong Agricultural University, Wuhan, China.

Molecular markers assays. DNA extraction and restriction fragment length polymorphism (RFLP) assays were conducted as described by Liu et al. (14). Total genomic DNA was extracted from fresh leaf tissues. In all, 324 RFLP markers were selected from two high-density rice genetic linkage maps (2,7) of S. D. Tanksley's group and the Rice Genome Project of Japan (RGP). Other probes for RFLP assays were subclones of BAC clones used in this research. A resistant bulk was made up by mixing equal amounts of DNA from 20 extremely resistant plants from the first F_2 population (lesion length <3 cm), and a susceptible bulk by mixing equal amounts of DNA from 20 extremely susceptible plants (lesion length >12 cm). The parents and the bulks were digested with 10 restriction enzymes (*Bam*HI, *Bgl*III, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*II, *Kpn*I, *Sca*I, and *Xba*I) and were used to detect the polymorphism and identify markers potentially linked to *Xa22(t)*. The RFLP markers showing polymorphism between the resistant and susceptible bulks were used to analyze the F_2 population.

SSR is a suitable choice considering the cost, labor, and genetic information provided for large-scale genetic and population studies. Two SSR markers (RM144 and RM224) were selected to identify recombination event around the target gene. RM144 and RM224 were mapped to the long arm of chromosome 11 (23) and flanked RFLP marker R1506 on different sides by >1.4 and 0.6 centimorgans (cM), respectively (22). Small quantities of DNA, extracted from 2-cm-long sections of fresh leaf seedling leaves, were used for SSR assays of the second population of 7,680 plants. SSR amplification was conducted as follows: The RM144 and RM224 primer pairs were pooled and used to amplify simultaneously in one reaction. The polymerase chain reaction (PCR) mixture of 20 μ l contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 $\times$ PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl), 0.185 μ M RM144 forward and reverse primers, 0.225 μ M RM224 forward and reverse primers and, 1 unit of *Taq* DNA polymerase. The PCR program consisted of one denaturation for 3 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1.5 min at

72°C; and a final extension step of 5 min at 72°C. After amplification, 16 μ l of PCR product was separated on a 2.5% agarose gel.

Linkage analysis. Linkage between molecular markers and the *Xa22(t)* locus was determined following the recessive-class approach (35), while the genetic distance from the relevant marker to the target region was evaluated by Mapmaker/Exp 3.0 (13) using the data of 248 random plants (Fig. 1A). The susceptible population of 404 recessive plants was used to identify tightly linked markers by analyzing the recombination events between the markers on the long arm of rice chromosome 11 and *Xa22(t)* (Fig. 1B). Plants associated with recombination events between the flanking SSR markers from the F_2 population of 7,680 individuals were used for constructing a fine structure map of the *Xa22(t)* region. The linkage index was evaluated as number of susceptible recombinant plants between the marker and the target region.

BAC library, filters, and isolation of BAC ends. Two BAC libraries cloned in pBeloBAC11 with blue and white spot selection were used for physical mapping. One BAC library from Teqing (*O. sativa* subsp. *japonica*) (34) consisted of 14,208 clones ($\approx$ 4.4-fold genome equivalents). Another BAC library from Minghui63 (*O. sativa* subsp. *indica*) (18) consisted of over 26,000 clones, or approximately eightfold genome equivalents. BAC library blotting and BAC clone isolation were conducted as described by Liu et al. (14). Thermal asymmetric interlaced PCR (TAIL-PCR) and Vector-Hexamer PCR were used to isolate BAC ends. TAIL-PCR, following the approach described by Liu and Whittier (15), included three rounds of PCR amplification. Vector-Hexamer PCR was performed as described by Herring et al. (8).

RESULTS

Fine genetic mapping of *Xa22(t)*. All of the F_1 plants from the cross ZZA $\times$ ZCL were resistant to pathogen strain PXO61 (data not shown). Segregation of resistant and susceptible plants fit a 3:1 ratio in a sample of 248 plants (178 resistant plants, 70 susceptible plants) taken at random from the F_2 population of more than 2,000 plants ($\chi^2 = 1.2097$, $P = 0.25$ to 0.50). In the F_3 families derived from the 248 random individuals, the segregation ratio of homozygous resistant families ($n = 60$), heterozygous resistant families ($n = 119$), and homozygous susceptible families ($n = 69$) was 1:2:1 ($\chi^2 = 1.0564$, $P = 0.50$ to 0.75). It was demonstrated that there was only one dominant resistance gene, *Xa22(t)*, determining the resistance against PXO61 in ZCL.

A total of 332 RFLP markers throughout the rice genome were assayed to identify polymorphism between the two parents and between the resistant and susceptible bulks. Approximately 30% of the RFLP probes revealed polymorphism between ZCL and ZZA when digested by 10 restriction enzymes. The polymorphism

Fig. 1. Southern blot analysis showing the marker L190 linked with resistant gene *Xa22(t)*. **A**, Random F_2 plants analysis for constructing linkage map flanking *Xa22(t)* locus; and **B**, susceptible F_2 plants analysis for identifying the recombination events between the marker and *Xa22(t)*. RB: resistant bulk; RP: resistant parent; SP: susceptible parent; SB: susceptible bulk.

ratios (number of RFLP probes showing polymorphism between parents in any one of 10 enzyme digestions compared with the total number of RFLP probes tested) on chromosome 3 and 6 were very low (12.9 and 5.9%, respectively), whereas the polymorphism ratios on chromosome 4, 5, and 11 were very high (51.4, 52.4, and 58.5%, respectively) (data not shown). It was indicated that the polymorphism ratios are different from different chromosomes. Only 12 markers on the long arm of chromosome 11 showed a difference between the resistant and susceptible bulks, indicating linkage between marker and the resistance gene.

Fig. 2. Fine-structure map of the bacterial blight resistance gene *Xa22(t)* with molecular markers in the telomeric region on the long arm of chromosome 11 in rice.

Fig. 3. Integrated contig map covering the resistance gene *Xa22(t)*. The core black bold bar indicates the *Xa22(t)* region and the markers are shown vertically. Markers in italics cosegregated perfectly with *Xa22(t)* using the population of 404 susceptible individuals, and the markers in bold represent the closest flanking markers of *Xa22(t)* locus in the genetic map. Bold bars represent contigs constructed by Minghui63 bacterial artificial chromosome (BAC) clones, and the slender bars represent Teqing BAC clones.

Among the 14 *Xa22(t)*-linked markers, 12 were RFLP markers from the two high-resolution genetic maps. All 14 markers were mapped within a 7-cM interval on a telomeric region on the long arm of chromosome 11 by assaying 248 random F_2 individuals. The marker R1506 was found to cosegregate with *Xa22(t)* by analyzing it both in the random population of 248 plants and the recessive population of 404 extremely susceptible plants that were selected from the same F_2 population. Two of the closest flanking markers (Y6855RA and G2132B) were linked to *Xa22(t)* with 0.4 and 0.7 cM, respectively (Fig. 2). The genetic distance between the two closely flanking markers was 1.1 cM, which was close enough to initiate chromosome walking to construct a physical map of the target region according to a ratio between physical and genetic distance of 244 to 280 kb/cM (3,28).

Physical mapping. To construct a physical map covering the *Xa22(t)* gene, five RFLP markers in the target region were used to screen two large-insertion BAC libraries, the Teqing BAC library and the Minghui63 BAC library. Twenty-four positive clones were selected from the Teqing BAC library by screening with probes R543, L1044, R1506, L190, and Y6855RA individually. Two clones, T32C23 and T37L15 (T indicates Teqing library), hybridized with R1506. Two subclones (SB6 and SC4) of T37L15 were identified to cosegregate with *Xa22(t)* by analyzing recombination events in the 404 susceptible F_2 plants. Two clones, T15B10 and T31F7, were selected by hybridization with SB6 and SC4. The end of T15B10 (T15B10F5) was found to cosegregate with *Xa22(t)* in the 404 susceptible F_2 plants. Fingerprinting analysis showed that T31F7 partially overlapped with the reverse (R) ends of T32C23 and T37L15, while T15B10 was contained in T37L15. T31F7 hybridized with G2132B, one of the closest flanking markers of *Xa22(t)*. A contig bridging R1506 and G2132B was constructed with Teqing BAC clones (Fig. 3, slender bars). New TQ BAC clones were not identified by the other end of T37L15.

Fig. 4. Fine mapping of *Xa22(t)*. **A**, Recombinant events analysis between molecular markers in the *Xa22(t)* region and *Xa22(t)* using 29 extremely susceptible recombinant individuals. **B**, *Xa22(t)* locus was mapped to one Minghui63 bacterial artificial chromosome clone M3H8. The number in the map indicates the amount of recombinant between marker and *Xa22(t)*.

TABLE 1. Recombinant events analysis between molecular markers in the *Xa22(t)* locus and *Xa22(t)* using 29 extremely susceptible recombination plants^y

Plant number ^z	RM144	L190	S12886	Y6855RA	3/7A10	M196-1	Sub11	X4-88	R1506	RM224
10	H	B	B	B	B	B	B	B	B	B
8	H	H	B	B	B	B	B	B	B	B
2	H	H	H	H	H	B	B	B	B	B
1	B	B	B	B	B	B	B	B	H	H
8	B	B	B	B	B	B	B	B	B	H

^y H = heterozygous bands of the susceptible and resistant parents at the marker locus and B = homozygous bands of the susceptible parent at the marker locus.
^z Number of recombination plants with identical genotype.

The contig bridging of R1506 and Y6855RA was not obtained with TQ BAC clones.

In order to construct the framework from R1506 to Y6855RA, the Minghui63 BAC library was screened with R1506 and Y6855RA. Nine positive clones were isolated (data not shown). Two clones, M3H8 and M11M24 (M indicates Minghui63 BAC library), showed hybridization with R1506. Seven clones hybridized to Y6855RA and one clone, M31B6, overlapped with clone M3H8 through the fingerprinting analysis. R1506 and Y6855RA were bridged by MH63 BAC clones (Fig. 3).

Finally, a contig covering resistance gene *Xa22(t)* was constructed by combining Teqing and MH63 BAC libraries (Fig. 3).

Recombination event assay. In the physical framework, recombination events descend sharply from flanking markers to cosegregating markers because of the relatively small population we first assayed. The target region cannot be narrowed down for sequencing and transferring. To further localize the target gene, 7,680 plants in the F_2 population were screened at the seedling stage for recombination events between two SSR markers (RM144 and RM224) that flanked R1506, one of the markers that cosegregated with *Xa22(t)*. In all, 220 recombinants plants that were heterozygous for one marker and homozygous for another marker were found by the SSR analysis. Of the 220, 29 susceptible recombinants plants were identified after inoculation with PXO61 at the booting stage and were chosen for determining the fine fragment location of *Xa22(t)*. Twenty were derived from recombination events between *Xa22(t)* and RM144 and nine between *Xa22(t)* and RM224. The recombinant individuals were characterized further by scoring with the more closely linked markers, L190, R1506, S12886, and Y6855RA (Table 1). Only two recombination events were identified between *Xa22(t)* and S12886 or Y6855RA, indicating that these were the closest marker to the *Xa22(t)* locus on the telomeric side of the gene (Fig. 4A). R1506 was the closest marker to *Xa22(t)* on the centromeric side of the gene, with only one recombination event identified. To localize *Xa22(t)* to a single BAC clone, a BAC-end subclone designated 3/7A10 from the BAC clone M3H8 (100 kb) was used to analyze the recombination events. The result showed that both recombinants between *Xa22(t)* and Y6855RA also were recombinant between *Xa22(t)* and 3/7A10. In addition, both 3/7A10 and R1506 hybridized to BAC clone M3H8. Both markers flank the *Xa22(t)* locus; this indicates that the *Xa22(t)* locus resides on the interval spanned by this 100-kb BAC, M3H8 (Fig. 4B).

DISCUSSION

Xa22(t) provides resistance to a wide spectrum of bacterial blight stains, and was localized to a small DNA fragment in this study. *Xa22(t)* should prove valuable when incorporated into elite rice cultivars after backcrossing into the parents of the prevalent rice hybrids or commonly used cultivars (4). Furthermore, the gene might be valuable if cloned and then introduced into breeding lines directly by transformation.

It has been quite difficult to construct physical maps through telomeric regions of rice chromosomes. There are many physical gaps in the telomeric region (3). We encountered difficulty in trying to bridge the region between the Y6855RA and R1506 markers with Teqing BAC library. The average ratio between physical distance and genetic distance of rice is 244 to 280 kb/cM, but the ratio can increase drastically in the regions next to telomeres or centromeres for the suppressed recombination in these regions (3). For rice blast resistant gene *Pi-ta²*, located in the centromeric region of chromosome 12, the ratio between physical distance and genetic distance was estimated to surpass 1,000 kb/cM (17). Our target gene lies next to the telomere in the long arm of chromosome 11 and the ratio reached 370 to 600 kb/cM.

Interestingly, many bacterial blight resistant genes (*Xa3*, *Xa4*, *Xa10*, and *Xa21*) have been mapped on the long arm of chromo-

some 11 (9,19,26,30,31). Two rice blast resistance genes, *Pi1* and *Pi44(t)*, also were mapped to the long arm of chromosome 11 (2). Using degenerate primers based on conserved motifs to amplify nucleotide binding site-leucine rich repeat (NBS-LRR) genes from the rice genome, several disease resistance gene homologues have been mapped close to the *Xa4* locus in the telomeric region of rice chromosome 11 (10,25). This indicated that the long arm of chromosome 11 is rich in resistance genes. Sequence of the 100-kb Minghui63 BAC clone showed that there are many resistance gene homologous sequences (X. L. Sun et al., unpublished data). The wide resistance spectrum of ZCL will be identified by transformation testing of the candidate fragment. This also should provide the opportunity to study the evolution of resistance genes.

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