Tagging *Xa-1*, the Bacterial Blight Resistance Gene in Rice, by Using RAPD Markers

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Summary

For identification of random amplified polymorphic DNA (RAPD) markers tightly linked to *Xa-1*, which is one of the bacterial blight resistance genes in rice, a near-isogenic line (NIL) for *Xa-1* and a recurrent cultivar were screened by RAPD primers. Two out of 280 primers produced DNA fragments tightly linked to *Xa-1*, namely U08₇₅₀ and Y03₇₀₀. No recombinant was detected between *Xa-1* and Y03₇₀₀, indicating that this RAPD fragment was closely linked to *Xa-1*. Y03₇₀₀ existed in IR-BB 1 and Kogyoku as a single copy sequence, but did not exist in IR 24. U08₇₅₀ was linked to *Xa-1* at a distance of 1.4 ± 1.4 cM. These RAPD markers will facilitate selection of *Xa-1* for breeding and will be starting points of gene cloning based on chromosome walking in near future.

Key Words : *Oryza sativa* L., gene tagging, RAPD, disease resistance, *Xanthomonas oryzae* pv. *oryzae*.

Introduction

Random amplified polymorphic DNA (RAPD) markers are highly useful molecular markers based on amplification of random DNA segments from the template DNA by polymerase chain reaction (PCR) using arbitrary primers (Williams *et al.* 1990, Welsh and McClelland 1990). RAPD markers are now commonly used for construction of linkage maps and tagging genes of interest, as well as restriction fragment length polymorphism (RFLP) markers.

In plants, several resistance genes against diseases (e. g. Martin *et al.* 1991, Michelmore *et al.* 1991) and nematodes (Klein-Lankhorst *et al.* 1991) have been tagged by RAPD markers using near-isogenic lines (NILs) (e. g. Barau *et al.* 1993, Martin *et al.* 1991, Paran *et al.* 1991) or bulked segregants (Michelmore *et al.* 1991). RAPD markers are more attractive than RFLP markers from the view point of breeding program, because the method is very simple to use. Moreover, in order to speed up the process of RAPD assay, rapid procedures for PCR have been developed (Edwards *et al.* 1991, Berthomieu and Meyer 1991, Yoshimura *et al.* 1992 a), making it unnecessary to purify the template DNA, the most laborious step, before the PCR procedure.

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama 1922) nom. rev. (*X. oryzae* pv. *oryzae*) is one of the most serious diseases of rice, and resistance to BB is one of the most important objectives for rice breeding. Thus far, the genetics of host resistance has been well studied and at least 19 resistance genes have been identified (Kinoshita 1991). NILs also have been developed for several resistance genes (Ogawa *et al.* 1988, 1991).

Xa-1 is one of the BB resistance genes that has a narrow pathogenic specificity. This gene conveys high resistance only to pathogenic race 1 of Japan, but not to any other races that have been identified in Japan and Philippines. According to the resistant reaction of the rice lines having this gene, *Xa-1* was categorized as a typical qualitative resistance gene (Ogawa and Khush 1989). However, little is known about the products of this gene or the mechanism of interaction between *Xa-1* and virulent pathogen. Map-based cloning of resistance genes will be one of the strategies to answer these questions. The first step towards map-based cloning would be the identification of molecular markers closely linked to a gene of interest. In this study, we report tagging *Xa-1* by RAPD markers using NILs and segregants.

Materials and Methods

Plant materials

To identify DNA fragments linked to *Xa-1*, a pair of NILs with and without the *Xa-1* gene, IR-BB1 and IR24, respectively, were used. IR-BB1 derived *Xa-1* from a japonica cultivar, Kogyoku and has undergone four backcrosses to an indica cultivar, IR24 and eight selfing generations. Kogyoku was also used to estimate the degree of polymorphisms between the parental cultivars of IR-BB1. F₂ population and F₃ lines derived from a cross between IR24 and IR-BB1 were used to investigate the linkage between *Xa-1* and polymorphic DNA fragments amplified with random primers.

Preparation of template DNA for RAPD analysis

DNAs were purified from fresh leaves of IR-BB1, IR24, Kogyoku, the F₂ and F₃ plants by using potassium acetate method (Dellaporta *et al.* 1983). For the segregation analysis between *Xa-1* and one of the polymorphic DNA fragments between IR-BB1 and IR24, crude extracts from leaves were also used instead of purified

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DNAs according to Yoshimura *et al.* (1992 a).

Bacterial inoculation

The F₂ population was tested for bacterial blight reaction with *X. oryzae* pv. *oryzae* strain T7174, a representative strain of pathogenic race 1 of Japan, to examine the segregation of *Xa-1*. For inoculum preparation, the bacterial cells were grown on potato semi-synthetic agar slants at 30 °C for 3 days. Inoculum was prepared by suspending the bacterial mass with sterile water at a concentration of about 10⁹ cells/ml. Two or three leaves of each plant were inoculated by the clipping method (Kauffman *et al.* 1973) at two months after sowing. Disease reaction was scored at 14 days after inoculation visually and classified into two categories, resistant and susceptible.

Primers and PCR reaction

Ten-base arbitrary primers were used for DNA amplification. The 280 primers were from the commercially available RAPD primer kits (Operon Technologies, Inc). Each primer had a GC content of 60 % or higher. The reaction conditions were as described by Yoshimura *et al.* (1992a). Amplified fragments were designated based on kit names, primer numbers and sizes of the fragments.

Linkage analysis

Recombination values between *Xa-1* and RAPD markers were estimated using the maximum likelihood equation (Allard, 1956), and the recombination values were converted into genetic map distances (cM) using the Kosambi function (Kosambi, 1944).

Southern hybridization of RAPD fragment

Two RAPD markers linked to *Xa-1* were cloned into plasmids vector pCRTMII (Invitrogen) using TA cloning kit (Invitrogen), and used as hybridization probes. Total genomic DNAs extracted from Kogyoku, IR-BB1, IR24 and F₃ lines were digested with the restriction enzymes *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV and *Hind*III. Ten µg of DNA per lane was separated by 0.7 % agarose gel electrophoresis and transferred onto nylon membranes (Amersham, Hybond N⁺). Southern hybridization analysis was carried out by using ECL, direct nucleic acid labelling and detection system (Amersham) following the protocol supplied by Amersham.

Results

Frequency of polymorphic fragments between Kogyoku and IR24

To estimate the ratio of polymorphic fragments that could be detected between parental cultivars of IR-BB1, DNAs extracted from Kogyoku and IR24 were subjected to the RAPD analysis using 20 primers, H01 to H20. About 100 discrete fragments ranging from 300 to 2500 base pairs (bp) were amplified from each cultivar and 40

of these fragments were polymorphic between them. When we assume that one band corresponds to one locus, this shows that about two loci can be screened by one RAPD marker in this combination.

Detection of polymorphic fragments between IR-BB1 and IR24

For identification of RAPD markers linked to *Xa-1*, IR-BB1 and IR24 were screened by 280 primers. Amplification without the template DNA was examined for 100 primers (H, I, O, S and Y) chosen randomly, and discrete or smear bands were amplified with 37 of the 100 primers. Almost all of these artifact amplifications were not detected when plant DNAs were added. When the bands corresponding to artificial ones were visible in the amplified products from plant DNAs, these bands were not included in the scoring of the fragments.

The majority of amplified DNA fragments from IR-BB1 and IR24 were identical in both the lines. However, using the primers, U08, W19, and Y03, one of the amplified fragments was appeared only in IR-BB1 but not in IR24. With the primers U01 and X02, fragments of different length, which were allelic to each other, were amplified both from IR-BB1 and IR24. This implies that these fragments are derived from the introgressed segments in IR-BB1.

Linkage analysis of Xa-1

To confirm the putative linkage between the polymorphic DNA fragments and *Xa-1*, the F₂ population of IR24/IR-BB1 was used for inoculation tests and for the RAPD analysis. The segregation ratio of resistant to susceptible to *X. oryzae* pv. *oryzae* strain T7174 was 63 : 24, fitting the expected ratio of 3 : 1 ($\chi^2 = 0.31$, $p > 0.50$).

As a preliminary examination of co-segregation between *Xa-1* and the RAPD markers, a small number of F₂ plants, including five resistant plants and five susceptible plants, were analyzed. Out of five pairs of polymorphic fragments from the primers U01, U08, W19, X02 and Y03, two fragments amplified by using primers U08 (5'-ACAGCCTGCT) and Y03 (5'-GGCGAAGGTT) were linked to *Xa-1*. The size of the fragments amplified by U08 and Y03 were 750 bp and 700 bp, respectively (see the parental lanes in Figs. 2 and 1A, respectively). The amplification products produced by U08 and Y03 are called U08₇₅₀ and Y03₇₀₀. The other three fragments from the primers U01, W19 and X02, were independent from *Xa-1*. Previously, Yoshimura *et al.* (1992 b) suggested that IR-BB1 had introgressed segments not only around *Xa-1* on chromosome 4 but also on chromosome 10. These negative polymorphic fragments would be derived from the introgressed segment on chromosome 10 in IR-BB1.

A larger F₂ population was used to estimate the genetic distances between *Xa-1*, Y03₇₀₀ and U08₇₅₀. The crude extracts from leaves of the same F₂ population were also used for analysis of Y03₇₀₀. With the primer

Fig. 1. Segregation of RAPD fragment Y03₇₀₀ in the F₂ population derived from the cross between IR24 and IR-BB1. Primer Y03 was used to amplify DNA fragments from purified DNA (A) and crude extract (B) of IR24 (P₁), IR-BB1 (P₂), Kogyoku (K) and F₂ population. The same F₂ plants are shown in A and B. Amplification products were separated by electrophoresis on a 2 % agarose gel and stained with ethidium bromide. Y03₇₀₀ is indicated by an arrow. The reaction of each plant to *X. oryzae* pv. *oryzae* inoculation is shown under the lanes. M ; molecular size markers (base pairs, bp), R ; resistant, S ; susceptible.

Fig. 2. Segregation of the fragment U08₇₅₀ in the F₂ population. DNA fragments were amplified using primer U08 from DNAs of IR24 (P₁), IR-BB1 (P₂) and the F₂ population derived from the cross between IR24 and IR-BB1. Each F₂ lane corresponds to the same F₂ in Fig. 1. U08₇₅₀ is indicated by an arrow. M ; molecular size markers (base pairs, bp).

Y03, all DNA fragments amplified from purified DNA (Fig. 1 A) were also amplified clearly from crude extracts (Fig. 1 B). For the analysis of U08, only purified DNAs were used (Fig. 2). The polymorphic fragments, Y03₇₀₀ and U08₇₅₀, were easily scored in the F₂ population. The segregation ratios based on the presence and absence of the polymorphic fragments were 61 : 23 ($\chi^2 = 0.25$, $p > 0.50$) for Y03₇₀₀ and 58 : 16 ($\chi^2 = 0.45$, $p >$

Table 1. Linkage analysis between *Xa-1* and RAPD markers

Gene pair	Segregation mode in F ₂				χ^2	Genetic map distance (cM)
	R+	R-	S+	S-		
<i>Xa-1</i> -Y03 ₇₀₀	61	0	0	23	84	95.51
<i>Xa-1</i> -U08 ₇₅₀	58	1	0	15	74	55.54

R : resistant, S : susceptible, + : present, - : absent.

0.50) for U08₇₅₀. These ratios fit the expected 3 : 1, therefore indicating that these DNA fragments were derived from rice nuclear DNA. The genetic distances between *Xa-1*, Y03₇₀₀ and U08₇₅₀ were calculated from the observed values of four classes (Table 1). No recombination could be detected between *Xa-1* and Y03₇₀₀ fragment, indicating that Y03₇₀₀ locus was tightly linked to *Xa-1*. Between *Xa-1* and U08₇₅₀ locus, one recombinant was found in 74 F₂ plants and the genetic distance calculated was 1.4 ± 1.4 cM.

Southern analysis of Y03₇₀₀ and U08₇₅₀

The usefulness of U08₇₅₀ and Y03₇₀₀ as hybridization probes was analyzed by Southern hybridization. The cloned fragments, Y03₇₀₀ and U08₇₅₀ were hybridized with restriction enzyme digested DNAs isolated from IR-BB1 and IR24. When Y03₇₀₀ was hybridized, a signal band was detected on only IR-BB1 but not on IR24, suggesting that Y03₇₀₀ was a single copy sequence and specific for the introgressed segment around *Xa-1* in IR-BB1 (Fig. 3 A). U08₇₅₀ showed one major and one minor bands on IR-BB1 and IR24 (Fig. 3 B).

Southern analysis was also performed on a part of the F₂ population (Fig. 3). DNAs were extracted from bulked F₃ seedlings, each derived from one of the 77 F₂ plants. When Y03₇₀₀ and U08₇₅₀ were hybridized on *Dra* I digested DNAs, the segregating patterns of the bands on autoradiograms fit the RAPD patterns (The numbers of the bulked F₃ DNAs in Fig. 3 correspond to the number of F₂ DNAs in Fig. 1 and 2.). This finding meant that the cloned fragments were the same DNA fragments as Y03₇₀₀ and U08₇₅₀. These cloned RAPD markers will be used as probes for map-based cloning.

Discussion

We have been studying tagging of BB resistance genes by using RFLP markers, and *Xa-1* was mapped between RFLP markers on chromosome 4 (Yoshimura *et al.* 1992 b). More closely linked and flanking molecular markers must be accumulated for use as selection markers in breeding programs and as starting points for map-based cloning of genes of interest.

About two fragments per primer were polymorphic between the japonica cultivar Kogyoku and the indica cultivar IR24 in this study. RAPD assay gives enough polymorphic DNA fragments to be used as molecular markers. In addition, the number of arbitrary primers is

Fig. 3. Southern hybridization of clones Y03₇₀₀ and U08₇₅₀ to DNAs extracted from IR24 (P₁), IR-BB1 (P₂) and the F₂ population digested with *Dra*I. Each F₂ lane corresponds to the same F₂ in Fig. 1. M; molecular size markers (Kilo base pairs, Kb).

almost unlimited and Klein-Lankhorst *et al.* (1991) showed that combining two primers gives new banding patterns. It should be possible to identify other RAPD markers tightly linked to *Xa-1* through further screening of primers.

As in the RFLP assay, more polymorphisms will be detected in RAPD analysis between rice cultivars that are genetically distant. However, some of these polymorphic bands, especially the less intense bands, were not useful in linkage analysis, because of inconsistent amplification in the segregating population. A small segregating population of 10 plants is enough for checking the stability of amplification of DNA fragments.

The RAPD marker Y03₇₀₀ will be a very useful marker to select *Xa-1* through breeding programs because it can be clearly amplified from crude leaf extract. Tagging agronomically important genes by RAPD markers that are amplified from crude materials makes the way to practical application of molecular markers in breeding programs.

Xa-1 has a narrow pathogenic specificity. *Xa-1* conveys high resistance only to pathogenic race I of Japan but it has susceptibility to all other races that have been isolated in Japan and the Philippines. However, little is known about the mechanisms of resistance and the gene products of BB resistance genes. Isolation of BB

resistance genes by map-based cloning would be a significant advance towards understanding of the mechanisms of host-pathogen relationship. Since Southern analysis showed that Y03₇₀₀ and U08₇₅₀ did not include repetitive sequences, these markers could be used as the starting points of chromosome walk to *Xa-1*. Further accumulation of the markers linked to *Xa-1* and construction of DNA libraries including *Xa-1* are in progress.

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