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RFLP Framework Map Using Recombinant Inbred Lines in Rice

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Summary
Recombinant inbred (RI) lines of rice (Oryza sativa L.) were developed from a cross of Asominori × IR24 by the single seed descent method. Seventy-one RI lines of the F0 and F2 generations were used for RFLP mapping. RFLP framework map constructed using the RI lines covered a distance of 1275cM containing 375 markers. All the twelve linkage groups were assigned to their respective chromosomes. The RI lines showed a distorted segregation in some regions of chromosomes 1, 3, 6, 11 and 12. Heterozygous loci in the lines still remained. Average frequency of the heterozygous region was 3.6% with a range of 0% - 19.3% in F0 and 1.5% with a range of 0% - 5.5% in F2. The RFLP map corresponded well to the previous maps constructed using F2 populations (Saito et al., 1991, Kurata et al., 1994) and the two previous maps were integrated in detail. The RFLP framework map and RI lines developed in this study will be useful for further accumulation of molecular markers and analysis of quantitative trait loci.

Key Words: Oryza sativa L., recombinant inbred lines, RFLP linkage map, map integration.

Introduction
Genetic maps using restriction fragment length polymorphism (RFLP) markers have been constructed for many higher plants (Paterson et al., 1991). In rice, several RFLP linkage maps have been reported based on RFLP clones by three research groups (Saito et al., 1991, Causse et al., 1994, Kurata et al., 1994). The RFLP markers and map information are contributing significantly to advances in the genetics and breeding of rice. However, since almost all of the maps were independently constructed using different segregating populations, it was difficult to directly integrate the maps and accumulate new information. Against this background, a permanent and common mapping population in which each line is genetically homozygous is required for further progress in molecular linkage mapping.

Recombinant inbred (RI) lines have several advantages over other single-meiosis segregating populations such as F2 or BC1F1 for linkage mapping. Since RI lines are genetically homozygous, they can be multiplied without further segregation and be distributed. Therefore, the researchers can use the same mapping population and all information for the markers. Additional markers scored on the same RI lines are automatically located on the existing map (reviewed by Burr and Burr 1991).

When RI lines are used as mapping population, co-dominant markers such as RFLPs and dominant markers such as random amplified polymorphic DNAs (RAPDs) can be mapped with the same efficiency (Reiter et al., 1992). The mapping of dominant markers is less efficient than that of co-dominant markers in an F2 population (Allard 1956).

An initial study for developing RI lines of rice and constructing a RFLP linkage map using the RFLP markers mapped by Saito et al. (1991) was carried out (Tsunematsu et al., 1993). In the current study, we aimed at the integration of the two RFLP linkage maps (Saito et al., 1991, Kurata et al., 1994) using the RI lines to obtain a permanent mapping population for the molecular markers.

Materials and methods

Population development
The RI lines were developed by inbreeding of the progeny of an F2 population derived from a Japonica-Indica cross of "Asominori" / "IR24" by the single seed descent method. A set of 165 F0 lines were obtained from 227 F2 individual plants. The reduction of the number of the lines in the F6 generation might be caused by the sterility occurred in the process of the advancement of the generations. Among them, 71 lines were randomly selected and a single plant per line was used for mapping. The RI lines of the F6 and F7 generations were used for RFLP mapping.

DNA extraction
Fresh leaves of a single plant in each RI line were collected and ground in liquid nitrogen or dry-ice. DNA was extracted from the ground tissues by the CTAB (Cetyltrimethyl ammonium bromide) method (Murray and
RFLP markers

RFLP markers previously mapped by Saito et al. (1991) (designated as XNpb numbers) and by Kurata et al. (1994) (designated as C, G, R, and Y numbers) were used. Rice genomic clones (designated as Ky numbers) newly prepared were also used. The genomic clones were derived from the rice cultivar IR24/Pst I library which contained inserts ranging from 0.5 to 4.0 kb. These Ky-numbered markers were the clones used in the initial study (Tsunematsu et al. 1993) with Q-number designation.

Southern hybridization

Radiolabeling system and enhanced chemiluminescence system were used for detecting RFLPs in the F6 and F7 generations, respectively. For the mapping of the F6 generation, the isolated DNA (2μg) was digested with six restriction enzymes (Bam HI, Bgl II, Dra I, Eco RI, Eco RV and Hin dIII), separated by 0.8% agarose gel electrophoresis and blotted onto a Hybrid-N Nylon membrane (Amersham) by capillary transfer in 20X SSC. After blotting overnight, the membranes were rinsed in 2X SSC, dried, and baked at 80°C for 2 hours.

DNA clone was radiolabeled with alpha 32P-dCTP by the random hexamer method (Feinberg and Vogelstein, 1983) using a multiprime DNA labeling system (Amersham). The labeled probes were individually hybridized overnight at 42°C in a hybridization solution (4X SSPE, 10% blotto, 50% formamide, 10% SDS, 0.5 mg/ml salmon DNA), washed twice at 42°C for 30 minutes each with 6X SSC containing 0.2% SDS and at 42°C for one hour with 2X SSC containing 0.2% SDS. After washing, the membranes were exposed to Kodak X-Omat AR film for 4-10 days at -80°C with an intensifying screen.

For the mapping of F7, 2μg of genomic DNA digested with eight kinds of restriction enzymes (Apa I, Bam HI, Bgl II, Dra I, Eco RI, Eco RV, Hin dIII and Kpn I) was electrophoresed on a 0.6% agarose gel. The gels were blotted onto a positively charged Nylon membrane (Boehringer Mannheim) by capillary transfer in 0.4N NaOH for 12 hours and the membrane was washed in 2X SSC, dried and baked at 120°C for 20 minutes. Almost all the probes were amplified by PCR and labeled with HRP (horseradish peroxidase) according to the protocol of ECL direct nucleic acid labeling and detection system (Amersham). Hybridized filters were detected by enhanced chemiluminescence on Fuji X-ray film for 1-3 hours.

Linkage analysis

Segregating markers were scored as either “A” (Asominori homozygote) or “B” (IR24 homozygote). Residual heterozygotes and the absence of data were scored as “-” (missing). Linkage analysis was performed by using MAPMAKER/EXP 3.0 (Lander et al. 1987). Two-point linkage analysis was performed. Pairs of loci were considered to be linked when the LOD score exceeded 3.0. Multi-point linkage analysis was performed to determine the order of markers within each linkage group. Recombinant frequencies and map distances were estimated using Plant Manager v 2.6.5 (Manly 1993).

Assessment of heterozygosity in RI lines

For each heterozygous RFLP locus, approximate segment size (in cM) was determined by halving the distance between two flanking markers on both sides of a heterozygous marker on the RFLP map. The total distance of heterozygous regions in each RI line was calculated by the sum of the segment sizes for each heterozygous locus. The percentage of the heterozygous regions in each RI line was estimated by dividing the total distance of heterozygous segments by the total map distance.

Results and Discussion

RFLP mapping

The genotypes of the markers were scored for each line based on the banding patterns on autoradiogram. An RFLP linkage map was constructed by using these data and MAPMAKER/EXP 3.0. The RFLP linkage map is shown in Fig. 1. The map contained 375 markers and the total map distance was 1275.4cM. The average distance between adjacent two loci was 4.4cM. All the 12 linkage groups were assigned to their respective chromosomes and no major discrepancy was found compared with the previous map (Saito et al. 1991, Kurata et al. 1994).

Comparison of the RFLP linkage map using RI lines with the previous maps

The RFLP map using RI lines (hereafter referred to RI map) was based on the RFLP markers which were previously mapped by Saito et al. (1991) and Kurata et al. (1994). To confirm the genome coverage provided by the RI map, the RI map was compared with the RFLP maps previously constructed. The results are as follows:

Chromosome 1; the RI map covered the linkage group between XNpb346 and XNpb216 described by Saito et al. (1991) and that between C112 and C970 by Kurata et al. (1994). RFLP markers, R3203, C3029C and C2340 (Kurata et al. unpublished), were newly mapped. Additional loci were mapped on the chromosome by using RFLP clones, C466, Npb297, R1468, C316 and Npb87, although these clones had identified the loci on another chromosome in the previous maps.

Chromosome 2; the RI map covered the full linkage group described by Saito et al. (1991) and the region between C1470 and R459 defined by Kurata et al. (1994). RFLP clone Npb89 detected a new locus outside of C1470 which was mapped at the distal end of the chromosome. A RFLP marker V83B (Kurata et al. unpublished) was newly mapped. Additional loci were mapped on the chromosome by using RFLP clones, C535 and C259.
Fig. 1. Rice framework RFLP linkage map constructed in this study. The map contains 375 markers and covers 1275.4cM. Marker designation followed that adopted by Saito et al. (1991) and Kurata et al. (1994). RFLP markers are not shown in Italic. Stippled regions along the chromosome represent the regions containing markers with skewed allele frequencies (P < 0.05). RFLP markers, C152 (chromosome 9) and C152B (chromosome 10), are rDNAs (18s ribosomal) RNA gene. The number in the Dis. column indicates the distance from the preceding locus. The cM column shows the map distance of the locus from the top of the linkage group.
Chromosome 3; the RI map covered the linkage group between XNpb164 and XNpb173 described by Saito et al. (1991) and that between G1318 and R1468A by Kurata et al. (1994). Additional loci were mapped on the chromosome by using RFLP clones, Npb212, C1454, R758, R411 and C161.

Chromosome 4; the RI map included all the linkage groups described by Saito et al. (1991) and by Kurata et al. (1994). Additional loci were mapped on the chromosome by using RFLP clones, Npb335 and C621.

Chromosome 5; the RI map covered the linkage group between XNpb329 and XNpb81 described by Saito et al. (1991) and that between C263 and C1447 by Kurata et al. (1994).

Chromosome 6; the RI map contained the full linkage group described by Saito et al. (1991) and the region between C1006B and C607 defined by Kurata et al. (1994). Additional loci were mapped on the chromosome by using RFLP clones, R1985 and C1677.

Chromosome 7; the RI map covered the linkage group between XNpb338 and XNpb22 described by Saito et al. (1991) and that between C1057 and R411 by Kurata et al. (1994). RFLP markers, XNpb50 (Saito et al. unpublished) and R2829 (Kurata et al. unpublished), were newly mapped. Based on the relationship of the three RFLP maps, the RFLP marker XNpb50 extended over the linkage group of chromosome 7.

Chromosome 8; the RI map covered the complete linkage group described by Saito et al. (1991) and the region between C83 and G278 (XNpb278) defined by Kurata et al. (1994). RFLP marker, R2976 (Kurata et al. unpublished), was newly mapped. Additional loci were mapped on the chromosome by using RFLP clones, C621 and C259.

Chromosome 9; the RI map covered the full linkage group described by Saito et al. (1991) and the region between C152 (rDNA: 25s ribosomal RNA gene) and G1445 defined by Kurata et al. (1994). Additional loci were mapped on the chromosome by using RFLP clones, G1314 and C796.

Chromosome 10; the RI map covered the linkage group between XNpb333 and XNpb127 described by Saito et al. (1991) and that between C701 and G127 (XNpb127) by Kurata et al. (1994). RFLP markers, R3285 and R1590 (Kurata et al. unpublished), were newly mapped. Additional loci were mapped on the chromosome by using RFLP clones, C152, C751, and R844. One of the fragments produced by probing rDNA (C152) was specific to the indica parent IR24 and mapped at the distal end of the chromosome. This result was in agreement with the findings based on chromosome in situ hybridization (Islam-Faridi et al. 1990).

Chromosome 11; the RI map covered the linkage group between XNpb181 and XNpb52 described by Saito et al. (1991) and that between R543 and C104B by Kurata et al. (1994). Three RFLP markers, R2918, C3029A and C718 (Kurata et al. unpublished), were newly mapped. Additional loci were located on the chromosome by using RFLP clones, C751, G1314, C952, C83, R1957 and R1466.

Chromosome 12; the RI map involved the whole linkage group described by Saito et al. (1991) and the region from C901 to G193 (XNpb193) defined by Kurata et al. (1994). Three RFLP markers, XNpb258 (Saito et al. unpublished), C3029B and C718B (Kurata et al. unpublished), were newly mapped. Additional loci were identified by using RFLP clones, Npb238, V24, Npb189, Npb24, C562 and Npb344.

These results indicated that the RI map covered almost all the regions defined by Saito et al. (1991) and Kurata et al. (1994).

Assessment of the RI lines

The average frequency of heterozygous regions was 3.6% with a range of 0% - 19.3% in the F₆ generation and 1.9% with a range of 0%-5.3% in the F₇ generation as expected (3.1% in F₆ and 1.6% in F₇) (Fig. 2). Only one line exhibited a high frequency of heterozygosity (19.3%) in the F₆ generation. This line contained a large heterozygous block especially on chromosome 2. However, the line did not show any heterozygosity in the F₇ generation. The other 70 lines did not show such a high frequency of heterozygosity in both F₆ and F₇ generations. The morphological characters, such as plant-height and heading date, were almost fixed within each line in the field observation. Genetical fixation of the RI lines had proceeded according to the expectation, although the lines had not achieved a complete homozygosity.

Segregation of parental genotypes at each locus

A population without segregation distortion in all the chromosomal regions is ideal for mapping. To examine the segregation distortion of markers, the deviation from the expected ratio of 1:1 at each RFLP locus was tested by chi-square test. The regions in which the markers showed the skewed segregation from 1:1 ratio at 5% significant level were found in 6 regions on chromosomes 1, 3, 6, 11 and 12 (Stippled region in Fig. 1). On chromosome 1, the RFLP markers between R210 and C970 exhibited a segregation distortion. Segregation of RFLP marker C953 showed a peak of distortion in this region with a 17.4% frequency of Japonica allele. The loci between C1677 and C563 exhibited a skewed segregation on chromosome 3 with the peak of skewness (22.6% of Japonica allele frequency) at the locus of XNpb392. Segregation distortion between XNpb27 and C962 was observed on chromosome 6 with the peak of distortion at G2028 (29.4% frequency of Japonica allele). There were segregation distortions in the region between C1350 and XNpb52 on chromosome 11. The peak of segregation distortion was found at C718 and the frequency of the Japonica allele was 31.4%. Skewed segregation of the markers was observed on both ends of chromosome 12. The RFLP markers between C901
and XNpb258 and that between C3029B and XNpb344-2 showed a significant skewed segregation. RFLP markers, C1069 and XNpb193 showed a peak of segregation distortion, respectively. The frequencies of Japonica alleles were 23.9% at C1069 and 14.7% at XNpb193.

RFLP linkage map using an F2 population derived from the same cross combination of the RI lines has been constructed. Some RFLP markers exhibited a segregation distortion on chromosomes 3, 6, 11 and in the region containing XNpb193 on chromosome 12 (unpublished data). These four regions were the same as those detected in the RI lines. At least in the above regions, the segregation distortions found in the RI lines appeared to be caused by the skewness in the F2.

The cause of the segregation distortion detected in the RI lines was not elucidated, but was speculated as follows. In chromosome 1, gametophyte gene ga-9 was reported (Maekawa and Kita 1985). Gametophyte genes, ga-2 (Nakagahra 1972) and ga-3 (Nakagahra et al. 1972), were also reported in chromosome 3. Several genes which possibly cause segregation distortion were reported in chromosome 6, namely, gametophyte gene (ga-I : Iwata et al. 1964), low crossability (Lcr : Sano 1992), hybrid sterility (S-5 : Ikehashi and Araki 1986, S-6 : Sano 1989), etc. A gametic lethal gene, gal (ll), was reported in chromosome 11 (Tomita et al. 1989). These factors may be responsible for the segregation distortion in the RI lines. In chromosome 12, segregation distortion in the progenies from Japonica-Indica crosses was not reported, indicating that the unknown causal factor(s) for segregation distortion occurred in chromosome 12.

**Duplicate segments between chromosomes 11 and 12**

Five among the 17 DNA probes mapped in the distal regions of chromosomes 11 and 12 detected duplicated loci. The loci, C3029, XNpb189, C718, R1957 and C104, were aligned in the same order on both chromosomes 11 and 12. The map distances of the duplicated linkage blocks were 7.5cM and 10.5cM on chromosomes 11 and 12, respectively. Nagamura et al. (1995) reported that the conservation of duplicated segments between rice chromosomes 11 and 12 was common in the varieties of *Oryza sativa*, as in the present study.

We developed rice RI lines and constructed a RFLP linkage map using them. Through this RFLP linkage map, the two RFLP linkage maps previously constructed (Saito et al. 1991, Kurata et al. 1994) were fully integrated. Based on the relationship between these three RFLP linkage maps, the RI map was considered to cover almost all of the regions defined by the previous RFLP maps (Saito et al. 1991, Kurata et al. 1994). The RFLP linkage map constructed in this study could be used as a framework map for the further mapping of molecular markers of rice. Molecular markers newly cloned can be immediately mapped on rice chromosomes using the RI lines and the map information. These mapping operations should contribute to further refinement of the map and the accumulated information should contribute to advances in rice genetics and breeding. Regarding the possibility of additional mapping, frequency of polymorphism between the parents is critical for users. Approximately 80% of the clones assayed in this study showed polymorphisms between the two parental varieties of the RI lines (data not shown).

The fact that RI lines constitute a permanent population is useful for the analysis of quantitative traits. The RI lines can be evaluated under different environmental conditions and the dominance effect can be ignored. Because a genotype is expressed by a line, rather than by an individual, error of measurement of the traits can be minimized. Studies on quantitative trait loci (QTL) using molecular markers have been carried out (Patserson et al. 1988) and demonstrated that RFLP mapping is an effective approach for identifying QTL (Tanksley et al. 1993). The segregation data for all the markers could be very useful for the analysis of the phenotypic variations observed in many traits of the RI lines. QTL analysis using the RI lines and the segregation data of the markers is now underway.

A set of seeds of the RI lines can be obtained from the Plant Breeding Laboratory, Faculty of Agriculture, Kyushu University and all the clones described here are available from the Rice Genome Research Program.
(RGP), Tsukuba, Japan, with clone numbers listed in Fig. 1. The map information including segregation data for the markers is also accessible by WWW server (http://bank.dna.affrc.go.jp) through the Internet.

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Literature Cited


