Screening of RAPD Markers Linked to the Photoperiod-Sensitivity Gene in Rice Chromosome 6 Using Bulked Segregant Analysis

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Abstract

Bulked segregant analysis was used to determine randomly amplified polymorphic DNA (RAPD) markers in a specific interval in the middle of chromosome 6 of rice for tagging the photoperiod sensitivity gene. Two pools of F2 individuals (japonica cv. Nipponbare and indica cv. Kasalath) were constructed according to the genotypes of three restriction fragment length polymorphism (RFLP) markers located at both ends and the middle of the targeted interval. Then another pair of pools were constructed based on the "graphical genotype," which was made with our high density linkage map. RAPD analysis was performed using these DNA pools as templates, and polymorphic fragments were detected and mapped. Using 80 primers, either singly or pairwise, we tested 2,404 primer pairs and established 14 markers tightly linked to the photoperiod sensitivity gene. The obtained RAPD markers were converted into sequence-tagged sites by cloning and sequencing of the polymorphic fragments and they can be used directly for construction of physical maps. This bulked segregant method can be applied for any species and any region of interest in which detailed linkage maps or physical maps are needed.

Key words: rice; bulked segregant analysis; sequence tagged-sites (STS); photoperiod sensitivity

1. Introduction

To produce molecular markers near a gene of interest or in the specific region of the genome, several strategies for pooling DNAs have been reported. Michelmore et al. selected lettuce F2 individuals resistant to downy mildew in one bulk and susceptible ones in another.1 Giovannoni et al. selected tomato F2 individuals according to the genotypes of RFLP markers flanking the genes responsible for regulation of pedicle abscission and fruit ripening and succeeded to set targets as intervals between two RFLP markers.2 Reiter et al. used recombinant inbred lines of Arabidopsis thaliana and constructed pools based on their genotype at all RFLP loci mapped on chromosome 1 for targeting markers to chromosome 1.3 Barua et al. used a doubled haploid population to identify markers linked to the Rhynchosporium resistance in barley.4 In these cases, random amplified polymorphic DNA (RAPD) method,5,6 a new procedure developed based on the polymerase chain reaction (PCR),7 was used for rapid and efficient detection of polymorphisms between bulked DNAs. These strategies produced useful markers efficiently, but the target interval tends to be longer than needed and some markers mapped too far to be utilized for map-based cloning. So setting of the target interval should be more precise, at the level of a few cM.

Photoperiod sensitivity has been considered as one of the agronomically important traits. A major photoperiod sensitivity gene, Se-1(Lm), has been localized in the middle of chromosome 68 and it is tightly linked to the blast resistance gene Pi-29 and to the glucose phosphate isomerase gene Pgi-2.10 Although there already exist some molecular markers linked to this gene,11 many more markers in this specific region are necessary to construct a physical map and for map-based cloning of the photoperiod sensitivity gene.

Quantitative trait loci (QTL) analysis was performed as a part of the Rice Genome Research Program (RGP) to localize several agronomically important genes using a cross of japonica rice cv. Nipponbare and indica rice cv. Kasalath. The photoperiod sensitivity is presumably coded in the middle of chromosome 6, at the Se-1 locus.12

In this study, we tried two rounds of bulked segregant analysis to produce RAPD markers around the presumed locus of the photoperiod sensitivity gene in the middle of chromosome 6. For the first round, DNAs of F2 individuals were selected according to genotypes of three markers flanking the region and the target was set at about

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Figure 1. Results of region-specific detection of RAPD markers by bulked segregant analysis. Two pairs of bulks were constructed targeting between L1092 and PgibA (30 cM, strategy 1) and between PgibA and L136 (14 cM, strategy 2), using DNAs of 11-13 individuals selected from 186 F2 population generated by crossing Japonica rice cv. Nipponbare and Indica rice cv. Kasalath. 15'16 Markers obtained by strategy 1 and strategy 2 are shown in circles or squares, respectively. Dotted line shows the presumed locus of photoperiod sensitivity gene Se-1. P5 and P3 are RAPD markers obtained by random mapping and reported already. 15

2. Materials and Methods

2.1. Plant DNA isolation and pooling

DNA was isolated from parents and F2 population as described previously. 15

For making bulks, at first F2 individuals which have the same genotype (A: Nipponbare or B: Kasalath) for three RFLP markers located at the each end (L1092 and PgibA) and the middle (L688) of the targeted interval were selected (see Fig. 1, strategy 1). Construction of DNA pools based on genotypes of several RFLP markers was reported by Giovannoni et al. 2 The bulks contained 11 (bulk A) and 12 (bulk B) individuals and the length of the targeted interval, that is between L1092 and PgibA, was 30 cM.

Secondly, for stricter setting of the targeted interval, we tried to select F2 individuals based on the graphical genotypes determined with our high-density genetic map.16 Desirable individuals to be contained in bulks are those whose genotypes are the same (A or B) only within the interval, and which have recombinations on either side of the interval (Table 2). The bulks contained 13 (bulkA) and 11 individuals (bulkB) and the target interval between PgibA and L136 was 14 cM (strategy 2).

2.2. Primers

Eighty 10-nucleotides primers named RA1 to RA80 were used in RAPD analysis. The sequences of RA1-60 were described previously. 15 The sequences of RA61-80 are shown in Table 1. Primers specific to the obtained RAPD markers were determined based on the sequences and listed in Table 3.

2.3. RAPD screening and mapping

The composition of the reaction solution and the conditions for PCR were described previously. 15 For the first screening, two pooled DNAs were used as a template and polymorphisms between them were detected. Eighty random primers described above were used both singly and pairwise to amplify two bulked F2 DNAs. Random pair-
The sequences of 10-nucleotide random primers used in this study. The sequences of RA1 to RA60 were described previously.15

| RA61  | AGCTTGCCCC  |
| RA62  | GAAGGCCGCTG |
| RA63  | GGGTACCGT   |
| RA64  | CGTCAAGCCC  |
| RA65  | GAGGCGGCTT  |
| RA66  | GACCGCTCAC  |
| RA67  | CGACGACCGA  |
| RA68  | CACCTTCGCC  |
| RA69  | GTCAGGGGAC  |
| RA70  | GGCGCGACTT  |
| RA71  | GAACCACCCC  |
| RA72  | CCAGCTAGGG |
| RA73  | CGGCTTGAGT |
| RA74  | CTGTGCTCCTG |
| RA75  | CGGTCAGAGT |
| RA76  | TCCTTCGCCG |
| RA77  | CGGACCGGTG |
| RA78  | AGCCAGACGC |
| RA79  | AGAGCAGCCC |
| RA80  | CATGACCGGC |

2.4. Determination of STS and STS-specific primers
Details of cloning, identification and sequencing of fragments, selection of primers and PCR with STS-specific primers were described previously.15

3. Results and Discussion
With bulks constructed by strategy 1, primary screening was performed using 572 combinations of primers and 5 RAPD markers were obtained (Fig. 1, presented in circles), however, two of them were mapped outside of the intended region. Two or three markers for selecting individuals may not be enough for precise setting of the target region. Actually, examining the genotypes of the F2 individuals selected in each bulk proved that the targeted region obtained was much larger than intended. Therefore we reconstructed F2 pools by strategy 2, using high-density graphical genotypes. This time, we tested 1,832 primer pairs and obtained 9 markers (Fig. 1, shown in squares). Although 4 markers were mapped slightly downstream of L136, most of the obtained markers were mapped inside the targeted interval.

In this case the resulting interval should be defined as the isogenic region among F2 individuals in the bulk; the region between the nearest recombination sites on each end of F2 individuals in each bulk. So it should be a little larger than the intended interval. The recombination sites of F2 individuals contained in each bulk in strategy 2 are shown in Table 2. Michelmore et al. showed that the foreign DNA can be detected as RAPD bands if it is contained only 1/5–1/10 quantity in the template DNA.1 In
Table 3. RAPD markers determined by bulked segregant analysis. Polymorphic fragments were cloned into pBluescript SK+ (Stratagene) and sequenced using a universal Dye-primer (Applied Biosystems) and an automated fluorescent DNA sequencer (Model 373A, Applied Biosystems). Based on the determined sequences, pairs of 20-mer primers specific to polymorphic fragments were selected using OLIGO version 4.0 (National Biosciences, Inc).

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Approx. length(bp)</th>
<th>Primers used</th>
<th>Primers found</th>
<th>DDBJ Acc.No.</th>
<th>Specific primer-1</th>
<th>Specific primer-2</th>
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<td>P108</td>
<td>450 (K)</td>
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<td>27+27</td>
<td>D50439</td>
<td>LP0287</td>
<td>LP0288</td>
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<tr>
<td>P123</td>
<td>290 (N)</td>
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<td>31+54</td>
<td>D50440</td>
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<td>Not determined</td>
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<td>D50441</td>
<td>LP0291</td>
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<td>50+?</td>
<td>D50442</td>
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<td>LP0296</td>
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<td>22+?</td>
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<td>78+?</td>
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<tr>
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<td>N.D.*</td>
<td>D50452</td>
<td>LP0447</td>
<td>LP0448</td>
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**Specific primer-1**
LP0287
CGACGGTAATACTGAAATA
AGAAACCGAGGCTCCTACAA
GATGTGATTGGCTCCCTACC
TATTTGTTCATCTTTTGTCG
TGTAGAGTCCAACCTGATGC
GGAGAAGAGAGGGAACAAGG
AATGAAACAGACCTTCTCAA
TCGGCTGGCTGTGTTATTTT
TACCGAAACTGTTATCCATA
ATTGATTCATTAGTGTCATA
CGACGGTAATACTGAAATA
GTCACGACGTAAACCTCTGC
CGACGGTAATACTGAAATA
TATTTGTTCATCTTTTGTCG

**Specific primer-2**
LP0288
TACCGAAACTGTTATCCATA
AGAAACCGAGGCTCCTACAA
GATGTGATTGGCTCCCTACC
TATTTGTTCATCTTTTGTCG
TGTAGAGTCCAACCTGATGC
GGAGAAGAGAGGGAACAAGG
AATGAAACAGACCTTCTCAA
TCGGCTGGCTGTGTTATTTT
TACCGAAACTGTTATCCATA
AGAAACCGAGGCTCCTACAA
GATGTGATTGGCTCCCTACC
TATTTGTTCATCTTTTGTCG

*1) Polymorphic fragment amplified from Kasalath DNA. *2) Polymorphic fragment amplified from Nipponbare DNA.
*3) Original primer sequences were not found in both ends of the cloned fragment.

this study, each bulk contains at least 1/10–1/13 quantity of DNAs of F2 individuals in which recombination occurred nearest to the interval end, and setting of target region was succeeded as described above.

We used 2% agarose gels for electrophoresis and the fragment size to be distinguished ranged approximately from 150 bp to 2 kb. The numbers of screenings needed for detection of one marker in one centiMorgan (cM) target region are calculated as 3,432 when the target interval was set between L1092 and PgibA (30 cM), and as 2,845 in the case of using graphical genotypes and with the target between PgibA and L136 (14 cM). The markers obtained by this method are RAPD markers, and it is difficult to use them directly for the construction of physical maps. However, they can be converted easily and reliably to STSs15 or SCARs14 which are very useful for map-based cloning. All of the polymorphic fragments of the 14 obtained markers were cloned into pBluescript SK+, identified by Southern hybridization and sequenced. Sequencings were performed from one end of the inserts and the full sequence was not determined for some of the long-sized fragments. The names of the original random primers whose sequences were found at the ends of the sequences are shown in Table 3. Eleven out of 14 markers were obtained by mixing of two random primers and at least 8 of them actually had different primers on each end of the fragment; then it was possible to detect these polymorphic fragments by simply mixing these primers in a RAPD reaction. The presence of another random primer in the reaction may increase the possibility of amplification of polymorphic fragments by dividing regions which are flanked by original primer but which are too long for PCR.

Specific primers were designed based on the deter-
Figure 2. Amplification of rice genomic DNA by specific primers selected based on the determined sequences of cloned RAPDs mapped on the targeted interval. PCR reactions were performed as described previously. The annealing temperature was usually 60°C except for P124 and P139 (55°C) and P137 (63°C). Nipponbare DNA (the first lane of each marker) or Kasalath DNA (the other lane of each marker) was used as the template. Primers used for each marker and sequence information of them are shown in Table 3. For electrophoresis, 4.5% polyacrylamide gel/0.5xTBE was used. Out of 13 markers, 6 were polymorphic between Nipponbare and Kasalath, two of which showed co-dominant features (P127 and P135). Primers for P135 amplified two slightly different sizes of fragments, suggesting the existence of another copy of the same sequence in the Nipponbare genome, whose locus has not yet been examined.

mined sequences (Table 3). Using STS-specific primers, a single product was amplified from all parental DNA (Fig. 2) except one (P123); three pairs of designed primers were tested to amplify only smeared products because of, presumably, highly repetitive sequences included inside the fragment.

Polymorphisms can not be always expected in PCR with STS-specific primers because original RAPD polymorphisms are likely to be due to mismatch at the primer sites.

In some cases, dominant polymorphisms were still observed with the PCR products using STS primers (P125, P129, P136 and P137) and co-dominant polymorphisms were observed for 2 markers (P127 and P135).

These clones were also tested as probes for RFLP analysis between Nipponbare and Kasalath DNA. Seven of them showed clear patterns and re-mapped on the same loci by RFLP with dominant (P125) or co-dominant data (P123, P126, P128, P130, P137 and P138). For these markers, screening of a YAC library by colony hybridization can clearly assign positive YACs. Other clones, however, showed multi-copy or smeared signals and further analyses could not be carried out (data not shown). For these clones, PCR with STS primers can be performed to select YAC clones to construct a physical map.

In this study, we obtained 14 markers altogether in the Se-I region of about 30 cM in the middle of chromosome 6 of rice. Using graphical genotypes, which was determined with a high-density genetic map, we were able to set the target interval very precisely. The bulked segregant method, especially with utilization of graphical genotypes, enables us to localize molecular markers anywhere in the genome and as many as we like. The width of the target region can also be chosen at will. We will apply this method for filling the gaps of our linkage map, some still in the range of several cM, or for tagging of other genes of agronomic importance whose locus is approximately determined by linkage analysis or by QTL analysis.

References


