Emphasized-RAPD (e-RAPD): a Simple and Efficient Technique to Make RAPD Bands Clearer

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The random amplified polymorphic DNA (RAPD) technique has been used widely for purposes such as the construction of linkage maps, QTL analysis, evaluation of genetic diversity, and parentage tests. However, some minor bands of RAPD display a low reproducibility and low reliability. We describe a simple and efficient method for making minor RAPD bands clearer. The first step of this method is to synthesize primers with nucleotides (A, T, G, or C) added to the 3′-end of the original primer sequences. The second step is to perform PCR using them and their combinations with the original primer. The final step is to conduct an electrophoresis analysis. The target bands can be emphasized, and needless background bands can be eliminated. The resulting emphasized RAPD (e-RAPD) bands are clearer and show a higher reproducibility than the original bands. This method has four advantages. (1) The developed e-RAPD bands can be used directly for marker-assisted selection (MAS). (2) The amount of DNA in the target band can be increased and, as a result, conversion to a sequence-tagged site (STS) marker is easy. (3) There is a lower risk of missing polymorphisms compared with conversion to a sequence-tagged site (STS) marker. (4) The method requires only primer synthesis and PCR, and thus after primer synthesis, the process can be completed within several hours. Easy, low-cost and time-saving detection system is a prerequisite for practical MAS for breeding purposes. For practical MAS, the method presented here using RAPD and conversion to e-RAPD is simpler and easier to apply than using amplified fragment length polymorphism (AFLP) markers or conversion to STS markers.

Key Words: emphasized-RAPD, RAPD, DNA marker, reproducibility, reliability, STS.
Original RAPD primer and design of primers for e-RAPD

The oligomer OPU-06 (sequence: ACCTTTGCGGG) (Operon, Alameda, CA) was used as the original RAPD primer. Synthesized primers with nucleotides (A, T, G, or C) added to the 3′-end of the original primer sequences were used for obtaining the e-RAPD band. Because the original RAPD primer was a 10-mer, the e-RAPD primer was an 11-mer. Primers with the added nucleotides were denoted by +A (sequence: ACCTTTGCGGGA), +T (sequence: ACCTTTGCGGT), +G (sequence: ACCTTTGCGGG) and +C (sequence: ACCTTTGCGGGG) primers. Similarly, further primers with nucleotides (A, T, G, or C) added to the 3′-end of the +G primer were synthesized, and were denoted by +GA (sequence: ACCTTTGCGGGGA), +GT (sequence: ACCTTTGCGGGGT), +GG (sequence: ACCTTTGCGGGG) and +GC (sequence: ACCTTTGCGGGG) primers, respectively.

PCR conditions

PCR solutions (10 µL total volume) contained 0.5 units of AmpliTaq DNA Polymerase (Applied Biosystems, Branchburg NJ, USA), the reaction buffer, 2.5 mM MgCl₂, 0.125 mM of each dNTP, 2 ng/µL template DNA and 2 µM primer DNA. The PCR temperature conditions were 1 minute at 93°C for denaturation, 1.5 minutes at 42°C for annealing, and 1 minute at 72°C for DNA extension. The PCR program included 40 cycles of the above conditions, 5 minutes at 93°C before the cycle to completely denature the DNA, and 10 minutes at 72°C after the cycle to allow complete double-strand DNA synthesis.

Results

Figure 1 shows the original RAPD pattern in the F₁ segregating population of tea. The arrow indicates the target band. This band was not clear and there were a few background bands. The primer or primer set was searched for e-RAPD to make the target bands clearer and eliminate background bands. In brief, PCRs were performed using the original primer, four kinds of 3′-additive primers (+A, +T, +G or +C primer) and different mixtures of 2 kinds of these primers. DNA samples from parents A and B were used as templates to confirm the polymorphism. Figure 2 shows the electropherogram of the PCR products used for the search for e-RAPD. Table 1 is an arrangement of Figure 2 with the accession numbers of the amplicon sequences. The original target band was denoted by ‘OT’. The bands with the same size appeared only if the +G primer was used alone or as one of the primers in the mixture. These emphasized target bands were denoted by ‘ET’. Notably, when +G was used singly, a strong band was visible: this band was denoted by ‘ET*’. When the +G primer was used with the F₁ segregating population, the clearer target band was prominent and most of the background bands disappeared (Fig. 3). Compared with the results in Figure 1, the results of e-RAPD with the segregating population clearly indicated that the bands obtained were the target bands. In addition, the polymorphic band ‘OA’ was emphasized by using a mixture of +G and +C primers (‘EA’ in Fig. 2). The previously neglected polymorphic band ‘OB’ was emphasized mainly by using the +C primer (Fig. 2, ‘EB’), and the neglected polymorphic bands ‘OC’ and ‘OD’ were emphasized by using a mixture of +T and +G primers (‘EC’) and a mixture of +A and +T primers (‘ED’). Unexpectedly, some bands that were originally faint or invisible became very clear (‘UE1’, ‘UE2’, and ‘UE3’). The annealing temperature for the emphasized target band ‘ET*’ was not critical between the initial temperatures of 42°C and 46°C (data not shown).

The emphasized target band (‘ET*’ in Fig. 2) was cloned and sequenced. The sequence was 1124 bp long and included the primer sequences at both ends. Figure 4 shows the structure of the amplicon of the e-RAPD band. The 3′-flanking nucleotides of the +G primer were A and C. Using further primers with nucleotides added to the 3′-end of the +G primer (+GA, +GT, +GG, and +GC primers), the target band appeared with a mixture of the +GA and +GC primers (Fig. 5). One of the unexpected highly visible bands (‘UE1’ in Fig. 2) was also cloned and sequenced. The sequence was 608 bp long and included the primer sequence in front and behind. There was no clear repeat sequence.
Discussion

This method enabled to achieve selective amplification based on the difference in the nucleotides of the 3'-flanking region of the primer annealing site. With this method, emphasis of the target band and elimination of background bands could be obtained very easily. The Tm value of the +C, +G primers or their mixtures was higher than that of the +A, +T primers or their mixtures. Therefore, the efficiency of the emphasized bands upon conversion to e-RAPD was higher when using the +G, +C primers and their mixtures than with +A, +T primers or their mixtures. Generally, e-RAPD bands were more reproducible and included fewer background bands than the original RAPD bands.

The annealing temperature can be increased depending on the Tm value of the primers. By utilizing differences in the second nucleotides of the 3'-flanking region of the primer annealing site, the e-RAPD band can be even more strongly emphasized. When one kind of primer for the original RAPD band is converted to e-RAPD, four kinds of primers are necessary. If two kinds of primers for the original RAPD band are converted to e-RAPD, eight kinds of primers are necessary.

This is an efficient method for making RAPD bands clearer, but it is not always successful, owing to primer dimer formation, similarity of the 3'-flanking nucleotides of the background bands, excessive and unexpected emphasis of the background bands and occasional loss of polymorphism.

However, this method has the following four advantages:

1. The clear band generated by e-RAPD can be used directly for marker-assisted selection.
2. The amount of DNA in the target band can be increased.

<table>
<thead>
<tr>
<th>Band name in Figure 2</th>
<th>Estimated band size(bp)</th>
<th>Accession number of sequence</th>
<th>Polymorphism and band origin</th>
<th>Primers or primer sets for the search for the e-RAPD band</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (target)</td>
<td>1120</td>
<td>AB085608</td>
<td>+</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
<tr>
<td>BG (background)</td>
<td>1110</td>
<td>B</td>
<td>+</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
<tr>
<td>A</td>
<td>800</td>
<td>A</td>
<td>+</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
<tr>
<td>B</td>
<td>760</td>
<td>B</td>
<td>±</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
<tr>
<td>UE1</td>
<td>610</td>
<td>AB085607 Non-polymorphic</td>
<td>−</td>
<td>+++ +++ ++ +</td>
</tr>
<tr>
<td>C</td>
<td>580</td>
<td>B</td>
<td>+</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
<tr>
<td>UE2</td>
<td>375</td>
<td>Non-polymorphic</td>
<td>±</td>
<td>+++ +++ ++ +</td>
</tr>
<tr>
<td>UE3</td>
<td>370</td>
<td>Non-polymorphic</td>
<td>±</td>
<td>+++ +++ ++ +</td>
</tr>
<tr>
<td>D</td>
<td>190</td>
<td>A</td>
<td>+</td>
<td>+G +C +A/T +A/G +A/C +T/G +T/C +G/C</td>
</tr>
</tbody>
</table>

−, ±, +, ++ and +++ indicate the absence or presence and the strength of the band, respectively. −−: band was not observed, ±±: faint band was observed, +: band was observed, ++: clear band was observed, +++: very clear band was observed.

*: Polymorphism was lost by using a mixture of +A and +T primers.
and, as a result, the conversion to an STS marker is facilitated.  
(3) There is a lower risk of missing the polymorphism than with conversion to an STS marker.  
(4) The method requires only primer synthesis and PCR, and thus, after primer synthesis the process can be easily completed within several hours.  

It has been recently suggested that the RAPD method may not be utilized for genetic analysis in the future (Ukai 2000). Certainly, many RAPD bands display a low reproducibility and reliability. In particular, minor RAPD bands are sensitive to the quality of the template DNA, the concentration of the PCR primer, the annealing temperature and even the type of equipment used for PCR. Generally, this low reliability of the RAPD method is caused by the difficulty in detecting minor bands. The e-RAPD system could alleviate these shortcomings of RAPD.

To obtain good markers for DNA marker-assisted
selection, we consider that it is easier and more practical to carry out mapping based on RAPD and conversion to e-RAPD than based on AFLP and conversion to an STS marker.

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Literature Cited


