Research Communication

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

Junichi Tanaka* and Fumiya Taniguchi

Breeding and Genetic Resources Team, Department of Tea Science, National Institute of Vegetable and Tea Science, National Agricultural Research Organization, 15451 Beppu, Makurazaki, Kagoshima 898-0032, Japan

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 an STS marker, if necessary, is easy. (3) There is a lower risk of missing polymorphisms than 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.

Introduction

For marker-assisted selection for breeding purposes, it is essential to obtain clear, allele-specific, tightly linked DNA markers. The density of the markers is very important in the search for tightly linked markers. Because dominant markers based on amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995) and random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990) techniques can be easily developed and are inexpensive compared with co-dominant ones, they are frequently used for mapping. However, AFLP technique is not practical for direct use in MAS and requires conversion to sequence-tagged site (STS) markers. However, the conversion is generally difficult, because AFLP bands contain low amounts of DNAs. In contrast, RAPD bands can be used directly for marker-assisted selection. Many RAPD bands, however, are not sufficiently clear to use for this purpose and show a low reproducibility and low reliability. Therefore, in many cases, conversion to STS markers is necessary, but usually very difficult. Sometimes, after cloning and sequencing of a target band, polymorphism is not observed, or sequences obtained are not derived from the original band due to the existence of closely located bands.

The low reliability of RAPD bands is usually caused by unclear minor bands. A method that makes minor RAPD bands clearer and eliminates background bands would be useful for direct marker-assisted selection and conversion to STS bands from RAPD bands.

We describe a simple method that makes RAPD bands clearer and eliminates background bands.

Materials and Methods

Plant materials and DNA extraction

Mature leaves of tea (*Camellia sinensis*) were used as DNA extraction sample. The clonal cultivar 'Sayamakaori' was used as parent A, and the clonal strain 'Kana-Ck17' was used as parent B. The F₁ progeny derived from a reciprocal cross between parents A and B was also used as the pseudo-testcross (Grattapaglia and Sederoff 1994) segregating population. DNA extraction was performed according to a method that uses diatomaceous earth and a spin filter (Tanaka and Ikeda 2002).

Communicated by K. Harada

Received January 30, 2002. Accepted July 1, 2002.

*Corresponding author (e-mail: tanajun@affrc.go.jp)

Original RAPD primer and design of primers for e-RAPD

The oligomer OPU-06 (sequence: ACCTTTGCGG) (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: ACCTTTGCGGA), +T (sequence: ACCTTTGCGGT), +G (sequence: ACCTTTGCGGG) and +C (sequence: ACCTTTGCGGC) 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: ACCTTTGCGGGA), +GT (sequence: ACCTTTGCGGGT), +GG (sequence: ACCTTTGCGGGG) and +GC (sequence: ACCTTTGCGGGC) primers, respectively.

PCR conditions

PCR solutions (10 μ l total volume) contained 0.5 units of Ampli-Taq DNA Polymerase (Applied Biosystems, Branchburg NJ, USA), the reaction buffer, 2.5 mM MgCl₂, 0.125 mM of each dNTP, 2 ng/ μ l template DNA and 2 μ M 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 608bp long and included the primer sequence in front and behind. There was no clear repeat sequence.

Fig. 1. Original RAPD patterns in the F₁ segregating population of tea. M: DNA size marker (ϕ X174/*HincII* digest).

(2) The amount of DNA in the target band can be increased

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

*: Polymorphism was lost by using a mixture of +A and +T primers.

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.

Acknowledgments

The authors thank Mrs. M. Iwata and Miss M. Sakamoto, assistants of the Breeding and Genetic Resources Team for their help, and Dr. Y. Takeda, head of the Breeding and Genetic Resources Team for critically reviewing the manuscript. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Rice Genome Project DM-1503).

Literature Cited

- Grattapaglia, D. and R. Sederoff (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: Mapping strategy and RAPD markers. *Genetics* 137: 1121-1137.
- Tanaka, J. and S. Ikeda (2002) The rapid and robust DNA extraction method from various plant species using diatomaceous earth and a spin filter. *Breed. Sci.* 52: 151-155.
- Ukai, Y. (2000) Genetic analysis at the genomic level: map and QTL. University of Tokyo Press, Tokyo. 350 p (in Japanese).
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Lee, M. Hornes, A. Frifiters, J. Pot, J. Peleman, M. Kuiper and M. Xabeau (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407-4414.
- Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.