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## Construction of PCR-based CAPS Markers for Rapid Genome Mapping

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Genetic maps consisting of restriction fragment length polymorphic (RFLP) markers are currently constructed in a variety of plant species. Since many important genes are first identified by their mutations, cloning of such mutated genes using a dense RFLP map (map-based cloning) is a powerful strategy, especially in *Arabidopsis thaliana*<sup>1,2)</sup> and rice<sup>3)</sup>, due to their small genome sizes. A common mapping strategy consists of isolation of total genomic DNA from individual F<sub>3</sub> families and analysis of RFLP by Southern hybridization.

Recently, new mapping techniques based on the polymerase chain reaction (PCR) were developed. CAPS (co-dominant cleaved amplified polymorphic sequences) markers depend on the ecotype-specific restriction patterns of DNA fragments amplified by PCR<sup>4)</sup>, whereas SSLP (simple sequence length polymorphisms) markers detect the difference of microsatellite sequences between ecotypes<sup>5)</sup>. Since PCR reaction requires much less DNA than Southern hybridization, F<sub>2</sub> plants can be used for DNA isolation and thus generation of many individual F<sub>3</sub> families is not necessary.

Although CAPS and SSLP markers are convenient for map-based cloning, the number of available PCR-based markers is not sufficient for high-resolution mapping (*e. g.* less than 1 cM). Therefore, it is often useful to create new CAPS markers based on existing RFLP markers. The construction scheme consists of (1) design of PCR primers, (2) survey of appropriate restriction endonucleases generating CAPS, (3) DNA isolation from individual F<sub>2</sub> plants, and (4) analysis of CAPS (Fig. 1).

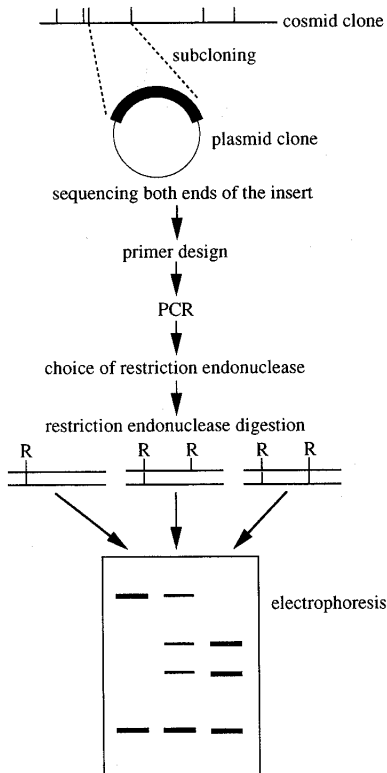
### 1. Design of PCR primers

When sequence data of existing RFLP markers are not available, markers must be partially sequenced after the end fragments of YAC or P1 inserts are amplified by PCR, or random fragments are subcloned from cosmid clones.

- (1) Digest a cosmid DNA marker with an appropriate restriction enzyme.
- (2) Subclone fragments with the size ranging from 1 kb to 4 kb<sup>6)</sup> in a plasmid vector (*e. g.* Bluescript).
- (3) Determine nucleotide sequences of the inserts from both ends.
- (4) Design and synthesize a pair of PCR primers according to the sequence data so that the corresponding genomic sequence in the range of 1-4 kb may be amplified by PCR. The ideal PCR primer pairs are 20-23 mer, have T<sub>m</sub> of around 60°C, include a G/C residue at the 3' end, and have no self-homology<sup>7)</sup>.

### 2. Survey of restriction endonucleases generating CAPS

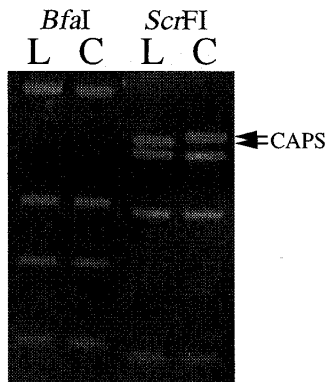
Since the size of PCR products is usually indistinguishable between two ecotypes, polymorphisms



**Fig. 1** Construction of a CAPS marker from an existing cosmid RFLP marker.

may be detected by the difference of restriction patterns.

- (1) Extract total DNAs from two different ecotypes (usually Colombia and Lansberg). Scale up the extraction procedure described in the section 3 below.
- (2) The reaction mixture of 100  $\mu$ l contains :
  - 1  $\times$  amplification buffer (supplied from Takara)
  - 125  $\mu$ M dNTPs
  - 500 ng primer 1
  - 500 ng primer 2
  - 5  $\mu$ l of DNA preparation (approx. 50 ng)
  - 1 U Ex *Taq* (Takara)<sup>9)</sup>
- (3) Amplify as follows :
  - denaturation for 30 sec. at 94°C
  - annealing for 1 min. at 55°C
  - polymerization for 1 min. at 72°C
  - cycle 40 times.
 The first annealing and the final polymerization steps are prolonged to 3 min. and 10 min. respectively.
- (4) Check the amplification by agarose gel electrophoresis. If a single major product is detected, proceed to the step 5.
- (5) Digest the products with restriction endonucleases recognizing four or five base pairs<sup>9)</sup>. We routinely test ten enzymes. When no CAPS is detected, additional ten enzymes are used.



**Fig. 2** Survey of restriction endonucleases generating CAPS.

The PCR product (0.95 kb) derived from Landsberg (L) and Colombia (C) was digested with either *BfaI* or *ScrFI*, and analyzed by 5% acrylamide gel electrophoresis. CAPS was detected with *ScrFI*.

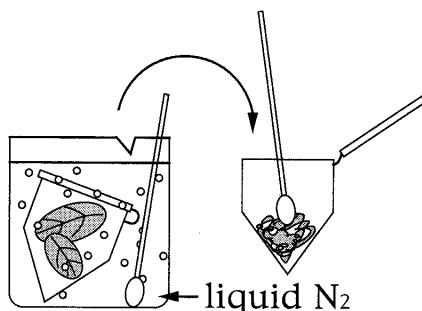
- (6) Analyze the restriction patterns by 4-5% acrylamide or 1-1.5% agarose gel electrophoresis.

A representative result is shown in **Fig. 2**. Genomic sequences of 0.95 kb derived from Landsberg and Colombia ecotypes were analyzed. Primers were designed based on the sequence data of an existing RFLP marker. While *BfaI* did not show any difference between two ecotypes, CAPS was detected by *ScrFI*.

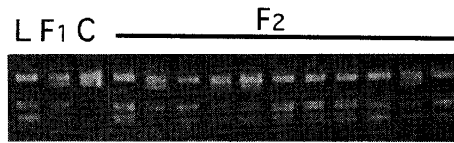
### 3. Isolation of total DNA

Although the PCR reaction is less sensitive to the purity of DNA than the restriction endonuclease reaction, crude DNA extracts often result in poor reproducibility of amplified band patterns. Therefore, we routinely employ the following protocol.

- (1) Sample two or three *Arabidopsis* leaves (0.1 g) into a 1.5-ml micro-centrifugation tube and freeze them in liquid  $N_2$ .
- (2) Grind them using a small ( $2 \times 150$  mm) spatula (**Fig. 3**).
- (3) Add 500  $\mu$ l 2 $\times$ CTAB buffer\*, and incubate at 60°C for 30 min.
- (4) Add 500  $\mu$ l chloroform: isoamylalcohol (24 : 1), and mix gently.
- (5) Centrifuge at 15,000 rpm for 5 min. at room temperature.
- (6) Transfer the aqueous phase into a new tube, and add 2/3 volume of isopropanol.
- (7) Centrifuge 15,000 rpm for 5 min. at room temperature.
- (8) Wash the pellet with 70% ethanol.



**Fig. 3** *Arabidopsis* leaves are frozen in liquid  $N_2$  and ground with a small spatula.



**Fig. 4** Segregation of the DFR locus in F<sub>2</sub> progeny from Landsberg (L) × Colombia (C) cross. F<sub>2</sub> plants were selected by a mutated phenotype originated from Landsberg ecotype.

- (9) Dissolve DNA in 200  $\mu$ l TE\*\* containing 20 ng/ml RNaseA (*e. g.* Sigma) and incubate at 37°C for 30 min.
- (10) Mix gently with 200  $\mu$ l phenol : chloroform : isoamylalcohol (25 : 24 : 1), and centrifuge the mixture.
- (11) Transfer the aqueous phase into a new tube, and add 1/10 volume of 3 M CH<sub>3</sub>COONa and 2 volume of ethanol.
- (12) Centrifuge 15,000 rpm for 10 min. at 4°C.
- (13) Wash the pellet with 70% ethanol.
- (14) Dissolve DNA in 100  $\mu$ l TE.

\* 2×CTAB buffer: 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% cetyl trimethyl ammonium bromide, 0.2% 2-mercaptoethanol

\*\* TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

#### 4. Analysis of CAPS

Individual F<sub>2</sub> plants are analyzed using the CAPS primer pairs.

- (1) The PCR reaction is carried out as described in 2-(2), (3). Scale down the reaction volume to 20  $\mu$ l.
- (2) Estimate the amount of the PCR product by agarose gel electrophoresis, if necessary<sup>10</sup>.
- (3) Restriction endonuclease reaction is carried out as follows :
  - 10  $\mu$ l PCR product
  - 2  $\mu$ l 10×restriction endonuclease buffer
  - 8  $\mu$ l H<sub>2</sub>O
  - 5-10 U restriction endonuclease
 Incubate samples under the appropriate conditions recommended for each enzyme.
- (4) Analyze CAPS by 4-5% acrylamide or 1-1.5% agarose gel electrophoresis.

A representative CAPS pattern is shown in **Fig. 4**. Two ecotypes, Landsberg and Colombia, their F<sub>1</sub> and 11 individual F<sub>2</sub> plants were analyzed by the CAPS marker DFR<sup>4</sup>. F<sub>2</sub> plants were selected by a mutated phenotype originated from Landsberg. Out of 22 alleles, 18 were shown to be originated from Landsberg, which indicated a linkage between the mutated locus and the DFR locus.

#### 5. Troubleshooting

- (1) PCR products appeared as a smear after electrophoresis.

The most common problems encountered in the mapping process are troubles in the PCR reaction. When the size of PCR product is heterogeneous due to mispriming, we routinely repeat the reaction with a higher annealing temperature. Also, longer annealing and polymerization duration may prevent amplification of shorter PCR products. When these alterations do not work, primers should be re-designed.

If amplified band patterns are different between DNA preparations, DNA must be extracted more

carefully.

(2) No polymorphism is detected.

Often CAPS is not detected even after testing many restriction enzymes. For example, we found six CAPS after testing eight potential DNA fragments. Nucleotide difference between Landsberg and Colombia ecotypes is estimated to be once in every 261 bases<sup>4)</sup>, and the investigated region of the genome may not contain any useful polymorphisms. It is best to give up the current fragment and try another.

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### References and Notes

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- 2) Nam, H.-G., J. Giraudat, B. den Boer, F. Moonan, W. D. B. Loos, B. M. Hauge, H. M. Goodman, 1989. Plant Cell, **1**: 699-705.
- 3) Kurata, N., *et al.*, 1994. Nature Genetics, **8**: 365-372.
- 4) Konieczny, A., F. M. Ausubel, 1993. Plant J., **4**: 403-410
- 5) Bell, C. J., J. R. Ecker, 1994. Genomics, **19**: 137-144.
- 6) Shorter fragments provide less possibility of finding polymorphisms and longer fragments may cause troubles in the PCR reaction.
- 7) Many softwares for the computer-assisted design of PCR primer are supplied(*e. g.* GeneWorks).
- 8) We observed that Ex *Taq* (Takara) is somewhat less sensitive to the purity of DNA preparations than Takara *Taq*.
- 9) Enzymes recognizing six base pairs, whose sites are included in the target sequences, may be also tested, when the entire sequence is known in one ecotype.
- 10) When the PCR product is too dilute, concentrate DNA by ethanol precipitation.