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Protocols

Cloning of AFLP Markers Detected by Fluorescence Capillary Electrophoresis

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Abstract. The available methods to isolate specific amplified fragment length polymorphism (AFLP) markers can be used only if markers are detected by radioactive labeling, silver staining, or ethidium bromide staining; these methods are useless if modern and automated genetic analyzers are used to detect AFLP markers by fluorescent labeling. We have developed a method that allows for isolation and cloning of specific AFLP markers obtained with a laser-induced fluorescence capillary electrophoresis system. This procedure has been tested on 5 *Arabidopsis thaliana* polymorphic AFLP markers, and the nucleotide sequences obtained from these cloned markers were identified and located in the *Arabidopsis* genome.

Key words: AFLP markers, capillary electrophoresis, cloning methodology, fluorescence labeling

Abbreviations: AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction; SRFA, selective restriction fragment amplification.

Introduction

The amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995) is frequently used for the identification of molecular markers because of its high efficiency, reproducibility, and reliability. Available AFLP protocols include different detection methods with nonlabeled, radioactive-labeled, or fluorescent-labeled selective amplification primers. The number and the consistency of the detected markers are similar for radioactive and fluorescent labeling but higher than that obtained when gel-staining procedures are used for nonlabeled primers. In addition, the use of fluorescent-labeled primers allows semiautomatization of the process because automated instrument systems capable of determining DNA base sequences have been adapted by their manufacturers to obtain DNA fragments of the size and quantity of AFLP markers.

However, the advantages of using fluorescent-labeled primers are negated if specific AFLP markers need to be isolated and sequenced because protocols for

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such a purpose are not available to date. In contrast, several methods have been published for radioactive labeling (Shan et al., 1999; Briard et al., 2000), silver staining (Cho et al., 1996; Chalhoub et al., 1997; Briard et al., 2000), and ethidium bromide staining (Scott et al., 1998). Here we present a methodology that allows the isolation and cloning of specific AFLP markers obtained by using a laser-induced fluorescence capillary electrophoresis system, such as the ABI Prism 310 Genetic Analyzer (Applied Biosystems), which can be easily implemented in similar models of genetic analyzers.

Materials and Methods

Plant materials and DNA extraction

Rosette leaves of 5-week-old *Arabidopsis thaliana* Ler-0 ecotype plants cultured in a greenhouse under controlled conditions (16/8 h photoperiod, 25°C) were excised, frozen in liquid nitrogen, and ground to a very fine powder. DNA was isolated by the main sodium dodecyl sulfate method described by Li and Chory (1998) but scaled down for use in Eppendorf tubes.

AFLP protocol

AFLP markers were obtained with the Plant Mapping Kit (Applied Biosystems) following the manufacturer's protocol for small genomes. DNA digestion with *Eco*RI and *Mse*I endonucleases (New England Biolabs) and ligation reaction of *Eco*RI and *Mse*I site–specific double-stranded adaptors were done in a single tube for 2 h at 37°C. Preselective and selective amplifications were carried out in a GenAmp polymerase chain reaction (PCR) system 9700 thermocycler (Applied Biosystems) following the manufacturer's protocol. Adaptors and preselective and selective primers designed specifically for analysis of small plant genomes (50-500 Mb) were used according to the Plant Mapping Kit protocol. The PCR products of selective amplifications were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer and detected by fluorescence of the 2 *Eco*RI site–specific selective primers used, EcoRI+TG and EcoRI+AA, which were 5′-labeled with carboxyfluorescein and carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein dyes, respectively. Unlabeled *Mse*I site–specific selective primers used were MseI+CAC and MseI+CAG. An internal size marker, Genescan Rox-500 (35-500 bp; Applied Biosystems), labeled with carboxy-X-rhodamine dye, was added, allowing the co-loading of different labeled reactions.

AFLP analysis

Data regarding selectively amplified DNA fragments were collected by a computer connected to the ABI Prism 310 that was running Data Collection 2.1 software (Applied Biosystems) and were analyzed with GeneScan 2.2 software (Applied Biosystems), which sized and quantified the detected fragments. GeneScan software was also used to compare the electropherograms from the analyzed plants in order to detect the polymorphic AFLP markers.

Specific AFLP marker cloning procedure

- Step 1—Raw data marker identification. To isolate a specific AFLP marker, we used GeneScan software to previously identify the marker in the raw data view of the analyzed data obtained from the corresponding AFLP reaction. Next, we reloaded the same reaction in the genetic analyzer to start a new electrophoresis. AFLP selective reactions stored at –20°C can be reloaded in the following 2 wk without appreciable loss of signal strength. By keeping open both windows on the computer screen, the real-time raw electrophoresis data window from the Data Collection software and the previous raw data window from the GeneScan software, it was easy to identify the moment when the specific AFLP marker was passing in front of the laser beam because the corresponding peak showed up on the right border of the real time window. The electrophoresis process was easily stopped at this precise time by opening, without any delay, the front door of the ABI Prism 310.
- Step 2—Capillary fragmentation. The capillary was then removed from the genetic analyzer and the window portion was cut with stainless-steel scissors in three 2-mm-long pieces. Each portion was placed into a different 0.2-mL Eppendorf tube containing 20 µL of selective AFLP amplification solution prepared with the selective primers corresponding to the AFLP marker to clone: 15 µL of AFLP Core Mix (Applied Biosystems), 1.0 µL of *Mse*I primer at 5 µM; 1.0 µL of *Eco*RI primer at 1 µM; 3.0 µL of Milli-Q water (Millipore). Each tube was labeled as T1, T2, or T3 according to the upper, central, or bottom original position of the corresponding window piece, and tubes were pulsed briefly on a centrifuge to ensure that the capillary fragments and the reaction solutions were located at the bottom of the tubes.
- Step 3—Selective amplification with capillary fragments. The T1, T2, and T3 tubes were placed in a thermocycler with a heated cover (Perkin-Elmer GenAmp PCR System 9700) for an AFLP selective amplification. Amplification conditions were as follows: 2 min at 94°C, 10 cycles of touchdown PCR (20 s at 94°C and 30 s at 66°C followed by 9 cycles with an annealing temperature decreased by 1°C per cycle, and 2 min at 72°C), 20 cycles of normal PCR (20 s at 94°C, 30 s at 56°C, and 2 min at 72°C), and 30 min at 60°C. These 3 amplification reactions were loaded in the genetic analyzer to determine which of them showed a better amplification of the AFLP marker to be cloned (Figure 1).
- Step 4—Unlabeled reamplification. The chosen T-number-selective amplification reaction was then diluted 10 times, and 4 µL was used to set up an unlabeled amplification reaction containing the preselective primers (15 µL of AFLP Core Mix, 1.0 µL of preselective primer pair, 4 µL of diluted T tube amplification). The following thermal cycler parameters were used: 2 min at 72°C; 30 cycles of 20 s at 94°C, 30 s at 56°C, and 2 min at 72°C; and 60°C for 30 min.
- Step 5—Cloning and colony insert analysis. The products from the unlabeled reamplification were ligated to a PCR cloning vector by the pGEM-T Vector System I Kit (Promega) and transformed into competent DH5-α *Escherichia coli* cells following standard procedures (Sambrook et al., 1989). Several positive transformant colonies were selected, and their plasmid DNA was isolated

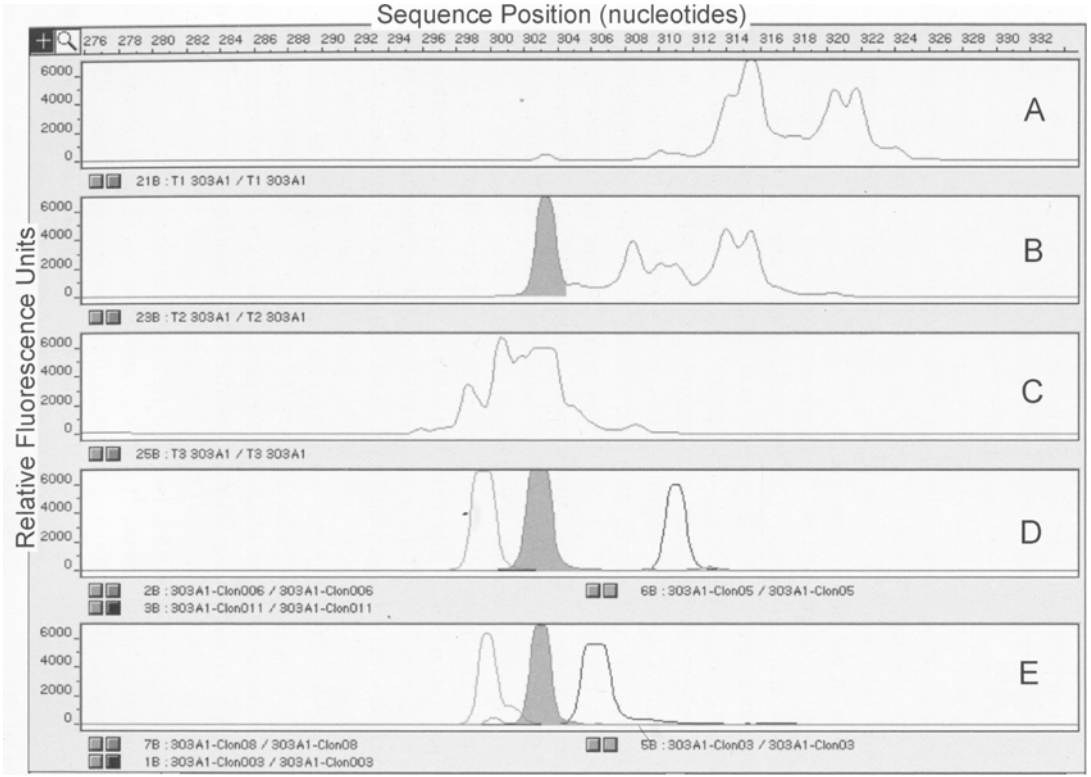

Figure 1. Electropherograms from capillary fragments and cloned AFLP markers. (A-C) Electropherograms of selective amplification reactions performed with the T1, T2, and T3 capillary window fragments obtained from an electrophoresis stopped in order to clone a 303-nt AFLP marker (filled peak). (D-E) Overlay of electropherograms of 6 selective amplification reactions performed with plasmid DNA corresponding to 6 transformant colonies obtained from T2 amplification; filled peaks indicate 2 colonies carrying the 303-nt AFLP marker.

by alkaline minipreparations (Sambrook et al., 1989). Finally, AFLP selective amplification reactions (see above) were set up with 0.05 μg of the different plasmid DNA samples to identify the colonies that carried the selected AFLP marker (Figure 1).

Plasmid DNA of the positive colonies can be used to obtain the nucleotide sequence of the AFLP marker cloned. In our case, overnight cultures of at least 2 positive colonies for each specific AFLP marker were sent to the nucleic acids sequencing facility of the University of León to obtain the sequence of the corresponding plasmid inserts.

Results and Discussion

Published methods for isolation of specific AFLP markers are based on direct observation of bands during autoradiography (Shan et al., 1999; Briard et al., 2000) or staining of acrylamide gels with silver salts (Cho et al., 1996; Chalhoub et al., 1997; Briard et al., 2000) or ethidium bromide (Scott et al., 1998), usually followed by extraction of DNA bands from the corresponding acrylamide gel. This approach is not possible if modern and automated genetic analyzers are used to detect the AFLP markers obtained with fluorescent labeling.

Genetic analyzers, such as the ABI Prism 310, are instruments based on laser-induced fluorescence capillary electrophoresis. In these systems, fluorescent dyes attached to single-strand DNA fragments are excited by a laser beam and emit light that is collected by a spectrograph when the DNA fragment travels through a small window located near the anodic end of the capillary. Therefore, if the electrophoresis is stopped at that precise moment, it is expected that a large number of molecules from the corresponding DNA fragment will be located just in the window portion of the capillary. This instant can be easily determined because the real-time light intensities at different wavelengths read at the capillary window (raw data) are shown on the screen of the computer controlling the instrument.

To clone a specific AFLP marker, it is necessary to stop the electrophoresis exactly when this marker is traveling through the capillary window. A comparison of the real time electrophoresis data against data from a previous electrophoresis of the same sample would ease this crucial step. We described above how to use the software provided with the ABI Prism 310 Genetic Analyzer for this comparisons and how to immediately stop the electrophoresis in this model, but it should be easy to implement a similar approach for other models of genetic analyzers.

The DNA located in the capillary window can be reamplified directly without an extraction step because the small amount of performance-optimized polymers that fill the capillary does not interfere with the DNA polymerase, in a way similar to that of Cho et al. (1996) by directly rehydrating SRFA bands excised from acrylamide gels as template in PCR reactions. The window can be easily cut from the capillary with scissors, but to avoid the reamplification of a large number of AFLP markers with similar nucleotide lengths, we fragmented the capillary window in 3 pieces.

A second reamplification step using unlabeled primers and the amplification products from the appropriate window fragment was necessary. The unlabeled

Table 1. Sequenced AFLP markers identified in the *Arabidopsis* genome.

Selective AFLP Primers[a]			*Arabidopsis thaliana* Chromosomal Location[b]			
EcoRI+2	MseI+3	Marker Size, nt	Chromosome (complete length, bp)	*Eco*RI Site	*Mse*I Site	Sequence Identities, %[c]
+TG	+CAC	166	III (23,470,805)	834,303	834,443	100
+TG	+CAC	204	I (30,432,563)	3,183,971	3,184,155	93
+TG	+CAC	303	II (19,705,359)	17,526,170	17,526,427	95
+TG	+CAC	355	I (30,432,563)	7,124,931	7,124,604	99
+TG	+CAG	202	IV (18,585,042)	14,063,941	14,063,767	99

[a]EcoRI+TG primer: 5′-GACTGCGTACCAATTCTG-3′; MseI+CAC primer, 5′-GATGAGTCCTGAGTAACAC-3′; MseI+CAG primer 5′-GATGAGTCCTGAGTAACAG-3′

[b]Restriction sites are indicated by the position of their 5′ end in the complete sequence of the corresponding *A. thaliana* chromosome

[c]Comparison from *Eco*RI site to *Mse*I site between the sequence of the AFLP marker and the published sequence for *A. thaliana* genome.

reamplification is needed because fluorescent dyes are large molecules located at the end of the AFLP fragments, which make efficient attachment to PCR cloning vectors difficult. The products of this second reamplification can be cloned by following standard procedures to obtain the nucleotide sequence of the selected AFLP marker.

Following the protocol described here, we have cloned and sequenced 5 polymorphic AFLP markers from *A. thaliana.* The sequence of the selective primers used to obtain the markers flanked all 5 sequences, and the internal sequences were identified and located in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000) by using BLAST searches of plant genomes (Table 1). These results indicate that this is a highly efficient method, although a relatively expensive capillary must be destroyed for each AFLP marker to be cloned. For this reason, we stored used capillaries that were close to expiration and used these capillaries for the AFLP marker cloning procedure.

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