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AFLP (Amplified Restriction Fragment Length Polymorphism)-Based mRNA Fingerprinting

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Several methods for the detection of transcripts that are differentially expressed in various tissues or at various developmental stages have been developed. Among these, methods for the fingerprinting of mRNA using PCR have been shown to be convenient and efficient in many situations [1-4], and two previously published methods [5, 6] employ the AFLP technology that was developed initially for the fingerprinting of genomic DNA [7]. For AFLP-based fingerprinting of DNA, genomic DNA is digested simultaneously with two restriction enzymes that recognize 6-bp and 4-bp sequences, respectively. The fragments were ligated to appropriate adapters and amplified with primers that anneal to the complementary sequences in the adapters. These primers have three selective nucleotides at their 3′ ends and only the 5′ end of the primer, which anneals to the adapters at the ends generated by enzyme that recognizes 6 bp, is labeled. This pattern of labeling reduces the complexity of bands during subsequent electrophoresis on a denaturing polyacrylamide gel. Application of AFLP technology to mRNA fingerprinting can be expected to have several intrinsic advantages [5, 6, 8] as compared to the differential display method with arbitrary primers, which is very sensitive to the reaction conditions and the quality of the template DNA [9-11]. However, one of the limitations of the AFLP-based fingerprinting of mRNA is the dependence upon the presence of the 6-bp recognition site of the restriction enzyme used, since such sites can be anticipated in only a small fraction of cDNA species.

In this protocol, we show a modified protocol for the AFLP-based fingerprinting of mRNA using *Taq* I, which recognizes a 4-bp sequence (**Fig. 1**). A combination of two primers, one with a single selective nucleotide at its 3′ end and the other with three selective nucleotides at its 3′ end and a radio-labeled 5′ end, results in a clear and highly reproducible pattern of bands on a denaturing polyacrylamide gel (**Fig. 2**).

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1. Templates for analysis of AFLP

Double stranded cDNA was prepared from poly

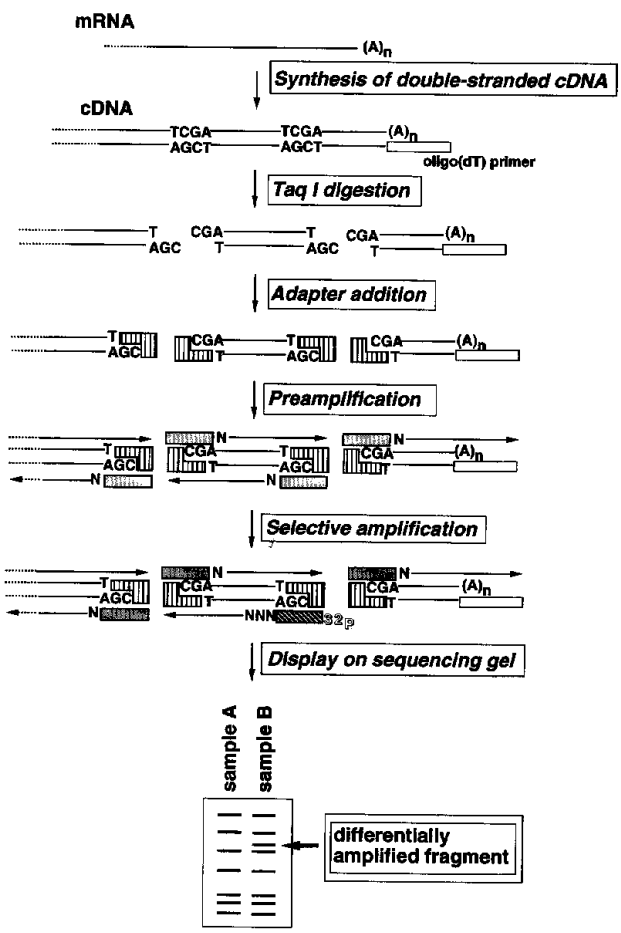

Fig. 1 A schematic representation of the strategy for the AFLP-based fingerprinting of mRNA. Double-stranded cDNA was synthesized by the standard protocol with oligo(dT) primers, and then it was digested with *Taq*I. The *Taq*I adapter was then ligated to each end of the fragments, and two-step amplification was performed with selective primers. Two types of selective primer were used: the first had a single selective nucleotide at its 3′ end, and the other had three selective nucleotides at its 3′ end and was labeled at its 5′ end. The combination of these primers allowed systematic screening of cDNAs and reduced the complexity of bands visualized on autoradiograms to a manageable level.

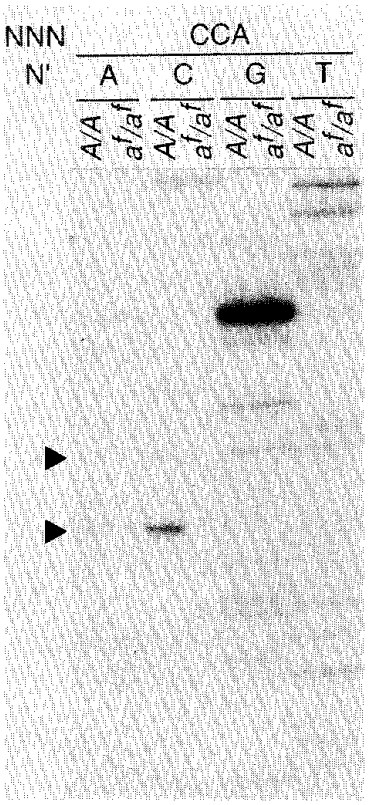

Fig. 2 A magnification of an autoradiogram of AFLP -based fingerprinting of mRNA.
Reaction products derived from buds of a plant carrying wild type *A* gene (*A*/*A*) and another plant carrying mutated *a* gene (a^t/a^t) of *Ipomoea purpurea*, respectively, were analyzed in two adjacent lanes for each combination of two selective primers. Two bands generated with the CCA/C primer combination (indicated by triangles in the left of the panel) are only seen in the lane of the *A*/*A* plant but not in the a^t/a^t plant. NNN, Sequences of selective nucleotides at the 3′ ends of labeled primers (in this case, CCA); N′, selective nucleotides at the 3′ ends of non-labeled primers.

$(A)^+$ RNA with a commercially available kit. The amount of the cDNA was estimated by counting the incorporation of radioactivity. One hundred nanograms of cDNA were digested with *Taq*I (10 U) and ligated to 175 pmol of *Taq*I adapters as described elsewhere [7]. The sequence of the *Taq*I adapters were as follows: top linker, 5′-GACGATGAGTCCTGAG-3′; bottom linker, 5′-CGCTCAGGACTCAT-3′. The 5′ end of the bottom linker was phosphorylated. Preamplification was performed in 20 μl of a reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.24 μM of two different *Taq*I+N primers (see below), 2.5 U of recombinant *Taq* polymerase (Takara Shuzo), and 2 μl of 10-fold-diluted ligation mix. The nucleotide sequences of *Taq*I+N primers, each of which had a single selective nucleotide at its 3′ end, were as follows: 5′-GATGAGTCCTGAGCGAN-3′ (where N represents A, C, G or T). PCR was performed for 20 cycles with the following temperature profile: 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C. The reaction mixture was then diluted 50-fold with TE (10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA, pH 8.0).

2. AFLP Reactions

*Taq*I +NNN primers, each with three selective nucleotides at the 3′ end, were labeled with ^{32}P as described previously [7]. The nucleotide sequences of *Taq*I+NNN primers were as follows: 5'-GATGAGTCCTGAGCGANNN-3' (where N represents A, C, G or T). Reactions were performed in 20 μl of a reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.24 μM *Taq*I+N primer, 1 μl of the reaction mixture for labeling of the *Taq*I+NNN primer, 0.5 U of recombinant *Taq* polymerase, and 5 μl of 50-fold-diluted preamplification mix. The temperature profile for PCR was as follows. The first cycle included 30 sec at 94°C, 30 sec at 65°C, and 1 min at 72°C. For the next following 12 cycles, the annealing temperature was reduced by 0.7°C per cycle. The final 23 cycles included 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C. After amplification, the reaction mixtures were combined with an equal volume of dye solution [98% (v/v) formamide, 10 mM Na_2EDTA, pH 8.0, 0.1% (w/v) bromo phenol blue, and 0.1% (w/v) xylene cyanol FF]. The mixture was heated for 3 min at 90°C and then quickly cooled to 4°C. A portion of the sample (2 μl) was loaded on a 4% (w/v) polyacrylamide/7.5 M urea sequencing gel (acrylamide: bisacrylamide = 30: 1). Electrophoresis was performed at constant power (70 W) for 2 hr. After electrophoresis, the gel was dried on filter paper (3MM; Whatman) and exposed to x-ray film (New A; Konica) overnight without an intensifying screen.

3. Cloning of amplified fragments

A piece of the dried gel containing the band of interest was cut out and soaked in 20 μl of H_2O for 10 min on ice. The sample was then heated for 15 min at 95°C and cooled again on ice. After a brief centrifugation, 10 μl of the supernatant were transferred to another tube. Reamplification of the recovered fragment was performed in 50 μl of a reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.4 μM *Taq*I+N primer, 0.4 μM *Taq*I +NNN primer, 2.5 U of recombinant *Taq* polymerase, and 2 μl of the solution of DNA. The temperature profile for PCR (30 cycles) was as follows: 30 sec at 94°C, 30 sec at 56°C,

and 1 min at 72°C. A portion (5 μl of the mixture after PCR was analyzed on a 2% (w/v) agarose gel. The remaining mixture was diluted 10-fold with TE and 1 μl of the diluted sample was used for ligation and transformation of *E. coli* with a TA Cloning Kit (Invitrogen).

4. Typical result

A typical result of the AFLP-based fingerprinting of mRNA is shown in **Fig. 2** We have analyzed the differential gene expression between red and white flowers of the common morning glory (*Ipomoea purpurea*) [8]. Reaction products derived from buds of the plants carrying wild type *A* gene (red flowers, *A*/*A*) and mutated *a* gene (white flowers, a^f/a^f), respectively, were analyzed in two adjacent lanes for each combination of two selective primers. Two bands generated with the CCA/C primer combination (the corresponding positions are indicated by triangles in the left of the panel) were only seen in the lane of the *A*/*A* plant but not in the a^f/a^f plant. Cloning of these fragments and subsequent analysis of the corresponding cDNA showed that these two fragments were derived from a single gene encoding chalcone synthase for anthocyanin biosynthesis. Comparing with the result of differential display performed in parallel using the same samples, the reproducibility of the AFLP-based fingerprinting of mRNA was shown to be extremely high [8].

5. Trouble shooting

(1) Faint signals or no reproducibility on autoradiograms.

Intactness of the RNA samples should be verified before and after the poly(A)$^+$ RNA selection. If the yield of the cDNA is low (less than 100 ng from 1 μg of poly(A)$^+$ RNA), other batches of RNA samples should be prepared.

(2) Many fragments with different sizes are amplified from a single band excised from gel.

Excised gel pieces usually contain various amounts of contaminating fragments that are amplified in the subsequent PCR. This is inevitable so it is necessary to perform Northern analysis with several independent fragments after subcloning.

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