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Note

A Fluorescence-based cDNA-AFLP Method for Identification of Differentially Expressed Genes

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Identification of differently expressed genes under specific tissues and/or environments provides insights into the nature and underlying mechanisms of cellular processes. Although cDNA-AFLP (Amplified Fragment Length Polymorphism) is a powerful method for analyzing differentially expressed genes, its use has been limited to the requirement of radioactive isotope use and the difficulty of isolating the bands of interest from a gel. Here, we describe a modified method for cDNA-AFLP that uses a fluorescence dye for detection and isolation of bands directly from a small size polyacrylamide gel. This method involves three steps: (i) preparation of cDNA templates, (ii) PCR amplification and differential display, and (iii) identification of differentially expressed genes. To demonstrate its utility and efficiency, differentially expressed genes during vegetative growth and appressorial development of *Magnaporthe oryzae* were analyzed. This method could be applied to compare gene expression profiles in a diverse array of organisms.

Keywords : cDNA-AFLP, gene expression analysis, fluorescence dye

Gene expression analysis using cDNA-AFLP (Amplified Fragment Length Polymorphism) has been used to identify differently expressed genes in both eukaryotes and prokaryotes (Dellagi et al., 2000; Gabriels et al., 2006; Lao et al., 2008; Sarosh and Meijer, 2007). This method is the same as DNA-AFLP except that the primary PCR template is complementary DNA of mRNAs rather than of genomic DNA. The cDNA-AFLP method has been successfully applied quantitatively to determine gene expression levels (Lao et al., 2008; Vos, 1998; Zhang et al., 2009), suggesting that cDNA-AFLP can be used to conduct comprehensive expression analysis at genome-wide (Breyne and Zabeau, 2001). Although cDNA-AFLP analysis is not as comprehensive as microarray-based methods, cDNA-AFLP analysis does not require a prior knowledge of the whole genome sequence and extensive expressed sequence tag (EST) of a target organism and has proven to be robust, reliable, and sensitive (Gabriels et al., 2006; Guo et al., 2006; Hmida-Sayari et al., 2005).

In the original AFLP method (Vos et al., 1995), (i) one of the primer was labeled with a radioisotope (^{32}P or ^{33}P), (ii) the resulting labeled amplified DNA fragments were electrophoresed in the large sequencing gels, and (iii) visualized via autoradiography. However, the use of a radioactive isotope raised contamination problems during the handling of samples, and band excision from the dried gel is often imprecise. To avoid these problems, non-radioactive detection methods of AFLP were developed (Chalhoub et al., 1997; Lin et al., 1999). Silver staining of the gel for the detection was described (Chalhoub et al., 1997), but many double-band patterns were observed, which hamper data analysis. Furthermore, this staining method exhibited lower sensitivity than that of the original. A chemiluminescent detection method (Lin et al., 1999) and the use of fluorescence-labeled primer (Roman et al., 1999) were also employed. However, the chemiluminescent detection method requires Southern hybridization and the use of fluorescence-labeled primer requires the synthesis of expensive primers and a specialized instrument to detect differentially expressed genes.

In this study, we developed a fluorescence-based detection system for cDNA-AFLP analysis. Ethidium bromide, which is the most widely used fluorescence dye to detect DNA or RNA in gels, is not only hazardous but also relatively insensitive. We used a more sensitive and safe staining dye called Vistra Green™ (Amersham RPN5786/5787, Little Chalfont, Buckinghamshire, UK), which can be used to stain DNA in both agarose and polyacrylamide gels and thus allows quick isolation of polymorphic bands directly from the stained gel. A small (10×10 cm) gel was also employed instead of a conventional sequencing gel to facilitate gel management.

We used two different RNA samples from *Magnaporthe oryzae* strain 70-15, which obtained from the Center for

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Fungal Genetic Resources (CFGR) at Seoul National University, Seoul, Korea. The strain was incubated on oatmeal agar medium (OMA, 5% oatmeal (w/v) and 2.5% agar powder (w/v)) at 25°C with constant fluorescent light to promote conidiation (Yi and Lee, 2008). Total RNA was extracted from appressorial and vegetative mycelia samples using easy-spin™ (DNA free) total RNA extraction kit (iNtRON Biotechnology, Inc., Sungnam, Korea) according to the manufacturer's instruction. RNA concentration was determined by Nanodrop (NanoDropTechnologies, Wilmington, DE, USA). Five μg of total RNA was used to synthesize cDNA using the first stranded and double stranded cDNA synthesis Kit (Stratagen, GIBCOBRL, Carlsbad, CA, USA). The double stranded cDNA product was cleaned up with phenol:chloroform:isoamyl alcohol (25:24:1) (Sambrook et al., 1989).

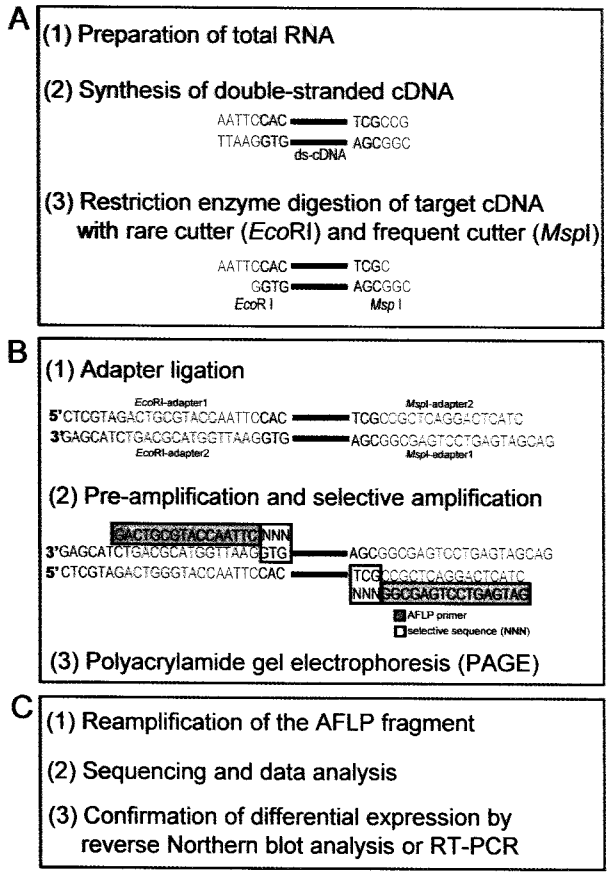

Fig. 1. Outline of the modified complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) procedure. A. Preparation of double stranded-cDNA and enzyme digestion with frequent cutter (*Msp*I) and rare cutter (*Eco*RI). After preparation of total RNA (1), double-stranded cDNA was synthesized (2). Red letters on right- and left-ends of cDNA show *Eco*RI and *Msp*I restriction enzyme sites, respectively. (3) As a result of double digestion with *Eco*RI and *Msp*I, both ends of the ds-cDNA are generated as sticky-end ands are ready to adapter ligation. B. Adapter ligation and primer design for PCR amplification. (1) Ligation of double-stranded *Eco*RI (blue) and *Msp*I (green)-adapters to the digested cDNA ends. (2) Pre-amplification without selective primer (gray box) and final selective amplification with selective primers (open box plus gray box), *Eco*RI-NNN and *Msp*I-NNN primers (Table 1), to allow detection of the transcript-derived fragment. C. Final steps to obtain sequence information from polymorphic fragments.

The resulting cDNA was digested with *Eco*RI and *Msp*I for 3 hr at 37°C. To prepare double stranded adapters using two complementary strands (Table 1), equal amount of 100 pmol *Eco*RI-adapter1 and *Eco*RI-adapter2, and 100 pmol *Msp*I-adapter1 and *Msp*I-adapter2 were ligated in a PCR machine by incubating 90°C for 10 sec and 25°C for 10 min ramping to 30 min (2.5°C/min). Digested cDNAs were ligated with 5 pmol of *Eco*RI and 50 pmol of *Msp*I double stranded adapters (Fig. 1) for 2 hr at 16°C. After ligation, the reaction mixture was diluted to 500 μl with TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Similar to the original protocol (Vos et al., 1995), PCR was performed in two steps: preamplification and selective amplification. The preamplification reaction was carried out using 25 cycles (94°C for 30 sec, 56°C for 1 min, 72°C for 1 min) with primers which correspond to 1 pmol of *Eco*RI and 6 pmol of *Msp*I adapters. Following the pre-amplification step, the product was diluted (50×) with TE buffer. Five μl were used for the selective amplification using 36 cycles including 13 touchdown cycles of 94°C for 30 sec, 65 to 56°C for 1 min, 72°C for 1 min comprising a reduction of the annealing temperature from 65°C to 56°C by 0.7°C in each step, which was then maintained for 23

Table 1. Primers used in this study

Primer	5'-3'
Adapter primers	
*Eco*RI-adapter1	CTC GTA GAC TGC GTA CC
*Eco*RI-adapter2	AAT TGG TAC GCA GTC TAC
*Msp*I-adapter1	GAG TCC TGA GTA GCA G
*Msp*I-adapter2	CGC TCA GGA CTC ATC
Standard primers	
*Eco*RI-standard	CTC GTA GAC TGC GTA CCA ATT C
*Msp*I-standard	GAC GAT GAG TCC TGA GCG G
Selective primers	
*Eco*RI-AGG	GAC TGC GTA CCA ATT CAG G
*Eco*RI-CAC	GAC TGC GTA CCA ATT CCA C
*Eco*RI-CAG	GAC TGC GTA CCA ATT CCA G
*Msp*I-C	GAT GAG TCC TGA GCG GC
*Msp*I-CT	GAT GAG TCC TGA GCG GCT
*Msp*I-CTC	GAT GAG TCC TGA GCG GCT C
*Msp*I-CTCA	GAT GAG TCC TGA GCG GCT CA
*Msp*I-CTG	GAT GAG TCC TGA GCG GCT G
*Msp*I-CTGC	GAT GAG TCC TGA GCG GCT GC

cycles of 94°C for 30 sec, 56°C for 1 min, 72°C for 1 min. Several combinations of primers were used for the selective amplification (Table 1).

After amplification, the samples were loaded in a polyacrylamide gel. The electrophoresis was carried out in a vertical gel electrophoresis system (Nippon EIDO Co. Ltd., Japan) using a 15% (running gel): 3% (stacking gel) polyacrylamide gel in TBE buffer system (0.1 M Tris-HCl, 0.1 mM EDTA, pH 8.0, and 89 mM Boric acid). Each sample was mixed with 5 μl of loading dye (10 ml of formamide, 10 mg of xylene cyanol FF, 10 mg of bromophenol blue and 0.1 ml of 0.5 M EDTA, pH 8.0) prior to gel loading. After loading 5 μl of the samples in each well, the gel was run at 70 volt for 12 hr. Gels were stained with 10,000 fold diluted Vistra Green™ for 10-30 min and then visualized under UV transilluminator.

Individual transcript-derived fragments (TDFs) of interest were excised from the gel on UV transilluminator and were soaked in liquid N_2. After grinding individual TDFs to a fine powder, 30 μl TE buffer was added. Reamplification of the recovered fragment was performed in a 20 μl reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 5 pmol *Eco*RI-NNN primers, 30 pmol *Msp*I-NNN primers, 1 unit of *Taq* polymerase (Takara, Tokyo, Japan), and 5 μl of the eluted TDFs. The temperature profile for PCR (25 cycles) was as follows: 30 sec at 94°C, 1 min at 65°C, and 1 min at 72°C. A portion (5 μl) of the reaction mixture was analyzed on a 1% (w/v) agarose gel.

Reamplified TDFs were mostly cloned into plasmid pGEM-T Easy® TA cloning vector (Promega, Madison, WI, USA) using a 8-well strip format. Two μl of reamplified product was ligated at 4°C for 8-12 hr in the presence of 3 μl of 2× ligation buffer, 0.13 μl of pGEM-T Easy vector and 0.26 μl of T4 DNA ligase in a total volume of 6 μl. This was routinely generated 10 to 50 transformant colonies per plate. One white colony was picked per plate. Direct sequencing of the TDFs was also performed.

Sequencing of the cloned TDFs was carried out using an Applied Biosystems sequencer ABI3700 (Applied Bio systems, Foster, CA, USA). Simultaneously, reverse Northern blot analysis was performed to confirm whether the TDFs correspond to differentially expressed genes or uniquely expressed genes under the given conditions. The *Escherichia coli* DH5a cells containing the plasmid clones were grown in 96 well plate at 37°C for overnight and were spotted on a 13×8.5 cm Hybond N+ nylon membrane (GE healthcare, Buckimghamshire, UK) on LB agar medium amended with 50 μg/ml of ampicillin using a High Density Replicating Tool (HDRT) containing 96 stainless steel pins of 1.14 mm diameter (Beckman part #267616, Fullerton, CA, USA). The plate was incubated at 37°C for 3 hr. The bacterial-growing membrane was treated by followings; 10% sodium dodecyl sulfate solution for 5 min, denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, neutralization solution (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 5 min, rinsed with 2 × SSC solution (3 M NaCl, 0.3M sodium citrate), air dried for 10 min, and then treated UV to crosslink DNA onto the membrane. To prepare probes for reverse Northern blot, the first-stranded cDNAs from mycelial and appressorial samples were used. Probes were individually labeled with ^{32}P-dCTP by random priming. The prepared membrane blots were hybridized with each probe sequentially as described previously (Park et al., 2003).

Using this method, AFLP fingerprints were generated from vegetative mycelial cDNA and appressorial cDNA of *M. oryzae*. The fingerprints were analyzed through (1) different fluorescence detection chemicals and (2) different combinations of *Eco*RI+NNN and *Msp*I+1 to 4 selective sequences (Fig. 1, Table 1). A number of polymorphic bands detected varied depending on fluorescence chemicals used and the primer combinations (Fig. 2, Table 1). Also, using Vistra Green™ exhibited a higher sensitivity and better resolution than ethidium bromide (data not shown).

A total of 13 primer combinations were used in this experiment. Typically, 15 to 30 bands ranging in size from 50 to 1,000 bp were obtained with each primer pair (Fig. 2). Although most bands were shared by the two samples, on average more than two bands per a primer set showed different intensity or unique presence for one of the samples (Fig. 2). The small size polyacrylamide gel (10×10 cm) made it easy to handle the gel and to isolate polymorphic bands after staining. Although a larger sequencing gel can resolve more polymorphic bands, it is difficult to handle and cut out the bands of interests directly from the gel. With increasing numbers of primer sets, more polymorphic bands can be identified.

The bands of interests were eluted in 30 μl of TE buffer and were then re-amplified for cloning and sequencing. Same primer sets were used for re-amplification to reduce the false positive. In most cases, it successfully reamplified the isolated fragment as a single band. The reamplified bands were cloned for sequencing and the level of expression was confirmed by reverse Northern blot analysis. Twelve colonies were selected for reverse Northern blot analysis. One of the clones showed strong hybridization signal to the appressorial cDNA probe, but not to the mycelia cDNA probe (Fig. 2B). The sequence of the clone matched with MGG15223.6.

This method can be also applied to compare rice cDNAs prepared with RNA samples isolated with and without chemical treatments. When 36 combinations of primer sets were applied, more than 100 differentially expressed genes were identified, supporting the broad applicability of this

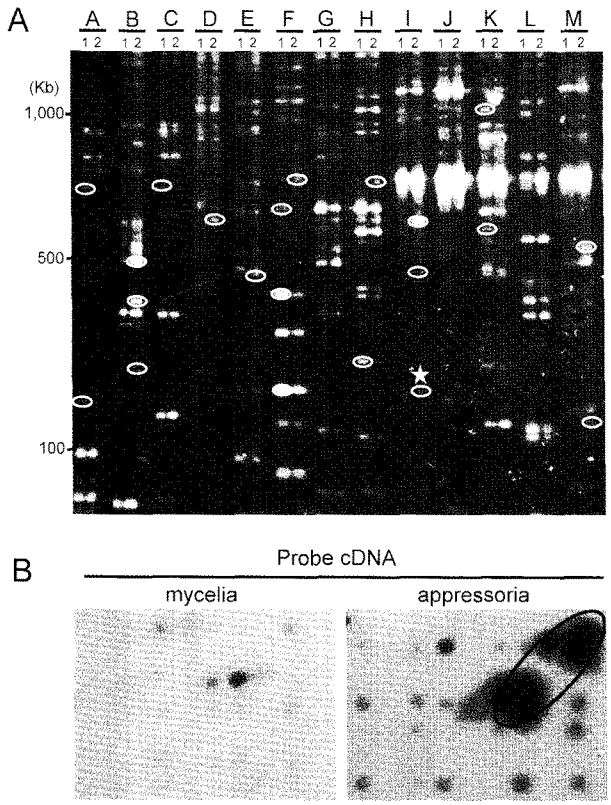

Fig. 2. A. cDNA-AFLP gel displaying gene expression patterns in RNA samples from two different conditions. Number 1 and 2 cDNAs were obtained from RNAs of *M. oryzae* strain 70-15 extracted from vegetative mycelia grown on complete medium and appressoria formed on inductive surface, respectively. A to M indicate the cDNA-AFLP amplification products by 13 primer sets (Table 1); Combination A, *Eco*RI-AGG/*Msp*I-CTCA, B, *Eco*RI-AGG/*Msp*I-CTG, C, *Eco*RI-AGG/*Msp*I-CTGC, D, *Eco*RI-CAC/*Msp*I-C, E, *Eco*RI-CAC/*Msp*I-CTC, F, *Eco*RI-CAC/*Msp*I-CTCA, G, *Eco*RI-CAC/*Msp*I-CTG, H, *Eco*RI-CAC/*Msp*I-CTGC, I, *Eco*RI-CAG/*Msp*I-C, J, *Eco*RI-CAG/*Msp*I-CT, K, *Eco*RI-CAG/*Msp*I-CTC, L, *Eco*RI-CAG/*Msp*I-CTCA, M, *Eco*RI-CAG/*Msp*I-CTG. Circles on the image indicate differentially expressed bands between two samples. B. Duplicate spots from the reverse Northern analysis. Left panel is hybridized with mycelia cDNA probe and right panel is hybridized with appressorial cDNA probe. An oval open box indicates strongly hybridized with appressorial probe but not with mycelial probe. The strongly hybridized clone was isolated from appressorial cDNA-AFLP using primer combination I (star mark on Fig. 1A).

method (data not shown).

We have developed a cDNA-AFLP method that involves a fluorescence-based labeling and small PAGE gel to identify differentially expressed genes. This method worked well for RNA samples isolated from both *M. oryzae* and rice. This method does not require radioactive isotope and expensive instruments. Moreover, bands of interests can be accurately isolated directly from the gel for further analysis.

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