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Full Length Research Paper

Development of a simple and powerful method, cDNA AFLP-SSPAG, for cloning of differentially expressed genes

Minsheng Wu

Department of Crop Science, North Carolina State University Raleigh, NC 27695, USA. Phone: 919-513-4647. Fax: 919-5157959. E-mail: mwu@ncsu.edu.

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A novel plant differentially expressed gene cloning technique called cDNA-AFLP-SSPAG was descrybed. Using this method, Differential mRNA expression was found between high and low heterosis groups of maize. Differential cDNAs were easily obtained from silver stained cDNA-AFLP separated on polyacylamide gels. The cDNA was then reamplified, cloned and fragments were sequenced. Sequenced clones were used as probes in northern dot blot analyses and library screening. Full-length cDNA was cloned from a library suggesting that the cDNA-AFLP technique combined with silver-staining polyacrylamide gels provide a novel and very powerful gene cloning method.

Key words: Cloning, maize, silver-staining polyacrylamide gels, GRF gene, heterosis.

INTRODUCTION

Since the AFLP technique was invented (Vos et al., 1995), it has been used widely for the construction of genetic linkage maps, genetic diversity analysis and gene cloning (Lorenzen et al., 2005; Terefework et al., 2001; Agrama, 2002). Recently, the AFLP-based transcript profiling protocol (cDNA-AFLP) was described. Significant advantages of cDNA-AFLP are a fragment-based technique for genome-wide expression analysis and the significantly higher sensitivity compared to other techniques including microarrays (Bachem et al., 1996). Furthermore, cDNA-AFLP is also a flexile tool that can be used even when genomic sequence information is not complete yet all the available sequence information can be utilized (Kivioja et al., 2005). Therefore, cDNA-AFLP has become one of the techniques for gene expression analysis in plants (Menges et al., 2003). Silver-staining of DNA in polyacrylamide gels had been used to visualize PCR-amplified fragments (Calvert 1995, Lohmann 1995). DNA products were readily recovered and reamplified from rapidly stained gels for making probes and sequence analysis (Chalhoub et al., 1997; Men and Gresshoff, 1998). Differential display techniques, though it has been used successfully in isolating genes in recent years (Li et al., 1994; Stephania et al., 1997), has the problem of false positives that lead to a increase considerable in labor (Aaron et al., 2001), which limits its application. However, the cDNA-AFLP technique is a highly sensitive method for cloning genes from a plant genome.

In this paper, using maize as a model system, the cDNA-AFLP technique combined with a silver-staining polyacrylamide gel technique was used to clone maize genes. We analyzed maize mRNA differential expression between high and low heterosis groups, and then used a differential fragment cloned using the cDNA-AFLP technique as a probe to screen maize cDNA library. The results shows that cDNA-AFLP in combination with silver-staining polyacrylamide gel technique is a powerful approach for the isolation of transcribed genes that are closely linked to the characteristics.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays L.)* leaves of seedling stage and elongation stage of tassel vegetative cone of maize inbreds P132, G489, Mo17 and its hybrid HHF1(P132 X G489), LHF1(G489 X Mo17) were harvested into liquid nitrogen and stored at –80ºC until used. (P132 X G489) has high heterosis, while (G489 X Mo17) has low heterosis.

Cloning procedure

Leaf or other materials in important stage of plant growth and development were selected. Total RNA was collected and treated

with Dnase. Reverse transcription was performed on the total extracted RNA to produce cDNA. The cDNA was then used in cDNA-AFLP PCR. The PCR products were loaded on sequencing gels and silver-staining polyacrylamide gels. Differential display expression bands were identified. The experiment was repeated to confirm results. Bands were excised from the gel for a second cDNA amplification using the same PCR conditions previously. The PCR product was used as a probe in a northern blot, clone sequencing, and full-length cDNA library screening.

RNA extraction and cDNA synthesis

Total RNA was isolated from leaves using the method described by Sambrook (1989). The extracts were purified by digestion at 37ºC with Rnase-free Dnase I (Pharmacia Biotech). Total RNA was cleaned with the RNeasy plant kit (Qiagen, Valencia, CA) according to the manufacturer's procedure, and the concentration was determined spectrophotometrically. Spectrophotometric analysis revealed high RNA quality with a A260/A280 ratio of 1.9-2.0. mRNA was prepared from total RNA with the PolyATtract mRNA Isolation system Kit (Promega) as described by the manufacturer. cDNA was synthesized from the mRNA using the cDNA synthesis system kit (Promega) according to the kit protocol.

Non-radioactive cDNA-AFLP reaction

Double-stranded cDNA (50 ng) was digested with 1 U Tag I and 1U Ase I in the presence of One Phor All (OPA) buffer (Pharmacia Biotech) and 10 µg BSA ml^{-1} for 3 h at 37ºC according to Vos et al. (1995). The digestion products (10 µl) were incubated with 1 pmol Tag I adaptor, 10 pmol Ase I adaptor, 2.5 µl 10 mM ATP, 0.5 µl NEB T4 DNA ligase in OPA buffer for 3 h at 37ºC. The adaptors were: 5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGCATGGAT-5(AseI) and 5'-GACGATGAGTCCTGAC-3' and 3'-TACTCAGGAC-TGGC-5' (Tag I). 2 µl of the ligation mixture was used as a template for the first amplification (pre**-**amplification) with the non-selective primer AseI (A00) (5'-CTCGTAGA-CTGCGTACCTAAT-3') and TagI (T00)(5'-GACGATGAGTCCT-GACCGA-3'). Pre-amplification was performed in a volume of 50 ul of 1X buffer containing 0.2 mM of each dNTP, 1.5 µl (50ng/ul) of each non-selective primer, 1U Taq DNA polymerase. PCR was performed under the following conditions: preliminary denaturation (2 min, 94ºC), then 29 cycles of denaturing (30 s, 94ºC), annealing (30 s, 60ºC) and extension (1 min, 72ºC). 5 µl pre-amplification product was checked on an ethidium bromide/1% agarose gel. The amplification products were diluted 50 fold and 5 µl used as a template for the second amplification reaction (selective amplification) with selective primers with 2-base extensions. Primer was complementary to the adaptors AseI and TagI. The AseI primers were A11 (extention AA), A(CT), A(GC), A(GT), and the TagI primers were T(AA),T(AT), T(AG), T(AC), T(TA). Selective amplification was performed in a volume of 20ul of 1X buffer containing: 0.2 mM of each dNTP, 0.6ul (50 ng/µl) of each selective primer, 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase. PCR was performed under the following conditions: preliminary denaturation (2 min, 94ºC), 1 cycle of 30s denaturing at 94ºC, 30 s annealing at 65ºC, 1 min extention at 72ºC; 12 cycles over which the annealing temperature was reduced from 72ºC by 0.7ºC each cycle; followed by 23 cycles of 30 s denaturing at 94ºC, 30 s annealing at 65ºC, 1 min extention at 72ºC. cDNA-AFLP product was electrophoresed through a 6% polyacrylamide denaturing gel.

Silver-stained polyacrylamide gel and cloning of cDNA-AFLP differential fragment

Gels were silver-stained according to Bassam (1991) with the following modification: 30 min fixation in 10% acetic acid, 4 min wash in distilled water (5 times), 30 min staining in a 0.1% silver nitrate, 0.05% formaldehyde solution, 5 s wash in distilled water, and revelation in a 4ºC pre-cooled solution containing sodium carbonate (30 g/l), formaldehyde (0.05%) and sodium thiosulfate (1 mg/l). Coloration was stopped in 10% acetic acid for 5 min. Gels were rinsed for 5 min (2 times) and dried overnight at room temperature.

After gel drying, cDNA-AFLP unique fragments were rehydrated for 20 min with 10 µl of distilled water, and cut from the polyacrylamide gel with a razor blade. Each gel slice was incubated in 40 µl of distilled water for 2 h at room temperature. 10 µl of eluent was then PCR amplified under the same conditions as for the selective PCR reaction. PCR product was separated on a 1.5% agarose gel and purified with a QIAquick Gel Extraction Kit (QIAGEN). PCR fragment was cloned using the Original TA Cloning kit (Invitrogen) according to the manufacturer's instruction.

Sequencing and analysis of cDNA-AFLP fragments

The nucleotide sequence was determined by using the 377 DNA sequencer (Applied Biosystems, CA). Sequence reaction was performed with the ABI prism Dye Primer Cycle Sequencing Core Kit from PE Applied Biosystems (Perkin Elmer). The FramePlot2.3 program was used for sequence analysis of cDNA-AFLP fragments. A BLAST-X search was used to identify the protein homologue.

RNA dot blot hybridization

Total RNA was transferred to a positively charged nylon membrane (BM) using a dot-blotting manifold and fixed to the membrane by baking in an oven at 120ºC for 30 min. 15 µg of RNA were blotted in each slot. cDNA probes were labeled with $^{[32P]}$dCTP by the random primer method (Feinberg and Vogelstein, 1983). The membrane was prehybridized with hybridization buffer (5x SSPE, 10 ug/ml denatured calf thymus DNA, 5% SDS) at 65ºC for 2 h. Then the membrane was allowed to hybridize with a radioactive probe in hybridization buffer at 65ºC for 16 h. After hybridization, the membrane was washed once with 5x SSC that contained 1% SDS at room temperature for 10 min and twice with 1x SSC that contained 1% SDS at 65ºC for 20 min. The signals were detected by autoradiography on X-Omat film (Kodak).

Construction and screening of maize cDNA library

cDNA library was constructed with λ UniZap vector kit (Stratagene, San Diego, USA) and *in vitro* packaging extract (Beringer Mannheim Biochemicals (BMB), Germany) according to manufacturer's protocols. cDNA library contained approximately $4X10^6$ clones, approximately 95% of which contained inserts.

Using cloned cDNA fragment as a probe, cDNA library was screened and positive clones were isolated. Full-length cDNA was analyzed by digestion with restriction enzymes and sequencing.

RESULTS AND DISCUSSIONS

Total RNA was extracted from maize leaves and the concentration determined by measuring absorbance at 260 nm (Sambrook et al., 1989). The integrity and DNA contamination of RNA extracted from maize leaves were evaluated by denature agarose gel electrophoresis. The protein contamination was examined by the A_{260}/A_{280} nm ratio (Sambrook et al., 1989). If the A_{260}/A_{280} nm ratio was lower than 1.8, a new phenol-chloroform extraction was done. In our study, the sample RNAs had a normal spectra (A260/A280=1.92) and could be used for cDNA

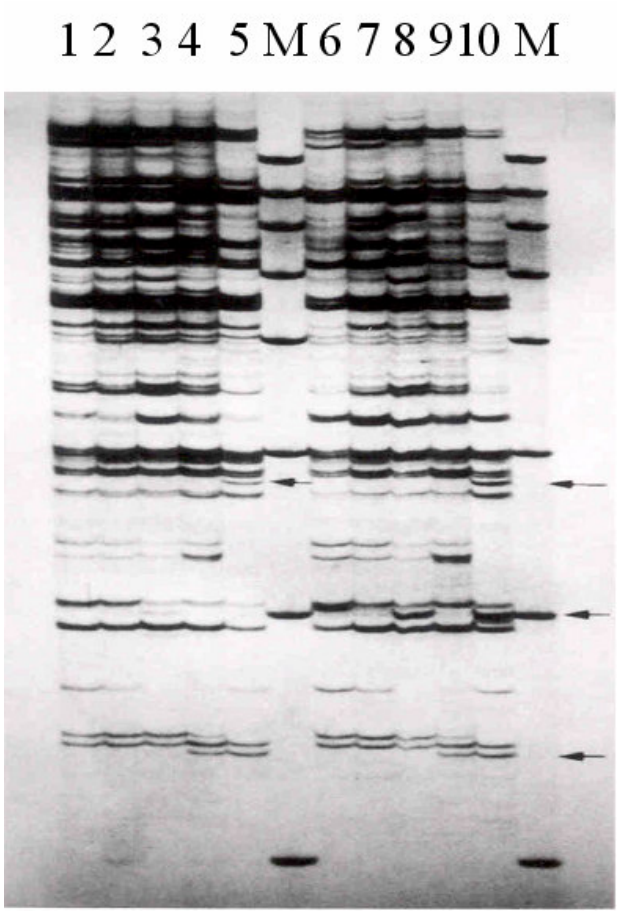

Figure 1. mRNA difference analysis of high and low heterosis combinations amplified with primer combinations (AseI +CT, TagI +AT). Seedling stage: 1, P132. 2, HHF1. 3, G489. 4, LHF1. 5, Mo17. Elongation stage of tassel apical cone: 6, P132. 7, HHF1. 8, G489. 9, LHF1. 10, Mo17. M,100 bp ladder DNA marker. The arrows indicated the differential bands

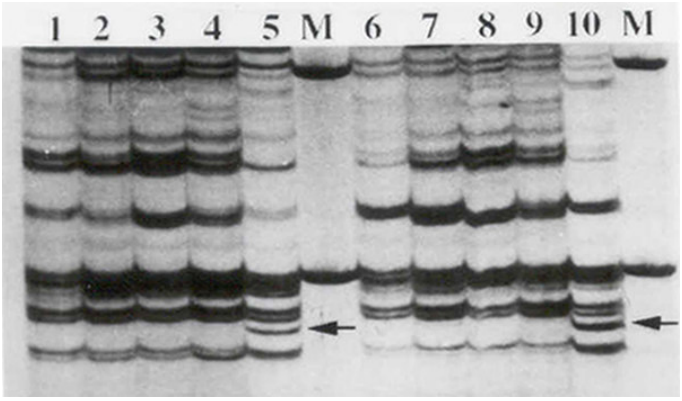

Figure 2. mRNA difference analysis of high and low heterosis combinations amplified with primer combinations (AseI +CT, TagI +AT). Seedling stage: 1, P132. 2, HHF1. 3, G489. 4, LHF1. 5, Mo17. Elongation stage of tassel apical cone: 6, P132. 7, HHF1. 8, G489. 9, LHF1. 10, Mo17. M,100 bp ladder DNA marker. The arrows indicated the differential bands.

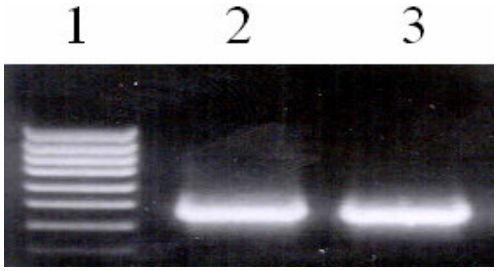

Figure 3. Second amplification of cDNA- AFLP fragment come from polyacrylamide gel. 1, 100bp ladder DNA Marker. 2, Mo17 at seedling stage. 3, Mo17 at elongation stage of tassel apical cone.

synthesis, cDNA-AFLP, cloning and sequencing. Gene expression of maize (*Zea mays* L.) for high heterosis combination (P132 x G489) and low heterosis combination (G489 x Mo17) at seedling stage and elongation stage of tassel vegetative cone were analyzed by cDNA-AFLP. We found that there were a lot of differential bands. Some suggested gene silencing. Some showed gene differential expression enhancement (Figure 1). Though mRNA was detected in the seedling stage and elongation stage of tassel apical cone of Mo17, it was different in two stages. The expression level of a gene at the seed-ling stage was higher than that at elongation stage of tassel vegetative cone (Figure 2). Furthermore, the gene was not expressed in their hybrids, which may be due to the interaction of the parent genes that led to gene silencing. Repeating the cDNA-AFLP experiment obtain-ned an identical map. These results showed that cDNA-AFLP technique could detect differential gene expression in maize.

To clone differential fragment from silver-stained polyacrylamide gel, the band from silver-stained polyacrylamide gel was cut. The eluted cDNA was used as template for PCR and then cloned into a vector. Figure 3 showthat PCR was successfully performed. The differential band was amplified. Furthermore, this was repeated four times and identical results were achieved (Figure 4). This showed one round PCR is enough for probe preparation and cloning. Sequence analysis showed that the size of the differential fragment isolated is 464 bp, which is enough to use as probe.

RNA dot blot hybridization has been used successfully for genetic analysis (Bodkin and Knudson, 1985b; Kowalik and Li, 1987; Nakagomi et al., 1989; Nakagomi and Nakagomi, 1991). In our study, we used this approach to determine the cloned gene. Hybridization of an RNA dot blot using a 464bp PCR derived fragment as a probe suggested the highest levels of expression in Mo17 (Figure 5). Mo17 displayed fairly uniform expression levels, showing approximately twofold higher signal intensity. RNA dot blot analysis revealed that differentially amplified cDNA fragments are derived from differentially expressed genes.

In order to obtain the full length gene, the cloned cDNA

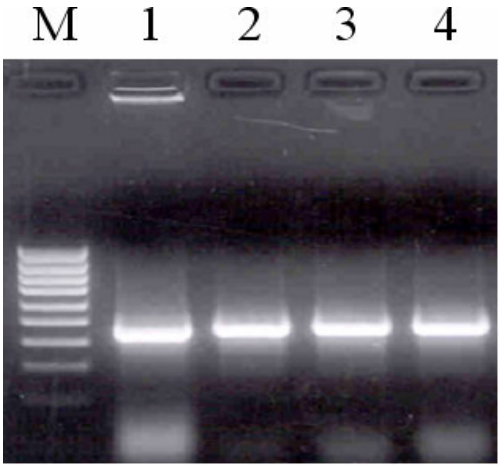

Figure 4. Repeat experiments for PCR. M.100 bp ladder DNA Marker 1-4: The same DNA of Mo17 isolated from polyacrylamide gel.

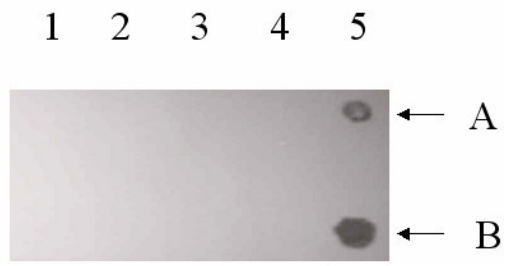

Figure 5. RNA dot blot hybridization. Total RNA (20 µg) extracted from maize was blotted onto nylon membranes. Membrane hybridized with cloning cDNA fragment labeled with ^{32}P. 1, P132; 2, HHF1; 3, G489; 4, LHF1; and 5, Mo17. The arrow A indicated at seedling stage. The arrow B indicated at elongation stage of tassel apical cone.

was labeled by the random-priming method using [α-^{32}P]-dATP and used to screen the amplified cDNA library made using RNA from elongation stage of tassel apical cone. A positive cDNA clone, maize13, was identified and sequenced. Nucleotide sequencing was carried out by the dideoxy-mediated chain termination method (Sanger et al., 1977) using a cycle sequencing kit (Pharmacia) and [α-^{32}P]-dATP. Initially pB SK (+) T3 and T7 primers (Stratagene) were used to sequence the 5' and 3' ends followed by complete sequencing of both strands by primer walking. Sequence analysis and multiple sequence alignment (PileUp) were accomplished by using the Genetics Computer Group (University of Wisconsin, Madison) and BLAST (Karlin and Altschul 1990) computer programs. Homologous genes or proteins were found in GenBank or other databases to discern the function from aspects of the sequence. The full-length cDNA (1083 bp) had an open reading frame of 840 bp, coding for a deduced protein of 280 amino acids. This gene is 98.6% identical to GRF1, and is 83% identical to 5'-end fragment of GF14-2 mRNA encoded by GRF1. Preliminary study indicated that maize 13 was a general regulatory factor (GRF). Ongoing experiments are designed to determine its role in heterosis. Identification of GRF gene provides a tool for understanding gene silencing at the cellular and tissue level.

Many researchers have been interested in understanding the mechanism of heterosis, but the exact mechanism for heterosis is unknown**.** Heterosis is the robustness seen in hybrids when different lines are crossed and result in higher yields than either of the parental lines would produce themselves. Heterosis involves complex interactions of several hormones and growth factors. In this context, gene interaction and gene expression plays an essential role in postnatal growth regulation (Jenkins, 1990**.**).

Recent study of the maize genome DNA sequence of two parents and its hybrid found heterosis of maize was closely related to regulatory factors from the genome (Song and Messing, 2003).

Based on the phenotypic variation and gene expression map and our data, we found some factor influencing gene expression. Finding associations between natural allelic variation and agronomical significant phenotypic variation could facilitate the deployment of natural genetic diversity for germplasm improvement. Therefore, comparing differential gene expression of maize at important growth and development stages is useful to screen for genes related to heterosis.

The cDNA-AFLP protocol has been improved so that only one fragment at most is obtained from each transcript (Breyne and Zabeau, 2001; Breyne et al., 2003). Furthermore, computer simulation technique provides a simple and inexpensive possibility to explore different options beforehand (Kivioja et al., 2005).

Based on the above research, cDNA-AFLP could provide a good method to clone plant gene. Our results showed that silver-staining based cDNA-AFLP is a time-saving, excellent alternative cloning methods. Therefore, it is a tool to clone plant DNA. cDNA–AFLP-SSPAG is a powerful tool to identify genes. No prior knowledge of gene is necessary. The method is relatively simple and straightforward. Other advantages of using cDNA-AFLP-SSPAG include avoidance of using radioactive labels, ease of band excision, and ease of obsevation differential display expression.

Conclusion

We successfully used cDNA–AFLP-SSPAG technique to study maize gene expression and cloned a gene related to maize heterosis. This result showed cDNA-AFLP-SSPAG was an alternative method for cloning of a maize differentially expressed gene. Experiments are in progress to clone more genes from maize.

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