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Rapid isolation of differentially expressed cDNAs from near isogenic lines of mungbean

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Abstract. We describe an application of cDNA libraries constructed from total RNA to isolate simultaneously many differentially expressed cDNAs between a pair of near isogenic lines of mungbean in a short period of time. All ten selected individual cDNA clones, containing inserts ranging from 1.1–1.9 kb, clearly showed positive Northern blotting. Nucleotide sequencing indicated that six of the clones were probably full-length cDNAs.

Keywords: Differentially expressed cDNAs; Mungbean; Near isogenic line.

Introduction

RNA fingerprint by arbitrarily primed PCR (Welsh et al., 1992) and differential display of RNA (Liang and Pardee, 1992) have been successfully used to isolate differentially expressed genes in two different states of many biological systems. Both techniques are powerful and require only small amounts of total RNA, but they also share some limitations. First, these methods produce a rather high rate of false positives, in which usually more than one cDNA species is present within one amplified band. Therefore, cloning a gene from the cDNA mixture is difficult. Consequently, obtaining cDNA clones that show positive results in Northern blotting is laborous and time-consuming. Various strategies have been tried to circumvent this problem (Mathieu-Daude et al., 1996; Zhao et al., 1996; Zhang et al., 1996; Shoham et al., 1996; Poirier et al., 1997). Secondly, it is impossible to obtain a full length cDNA by these methods, because of the limitation of the DNA sequencing gel that allows determination of only a maximal length of 500 bp. A full length cDNA has to be isolated from a cDNA library or by means of a RNA-anchored cDNA extension (RACE) method. Thirdly, these methods use radioisotopes. In this paper, we describe a rapid method based on the CapFinder Kit to simultaneously isolate many differentially expressed cDNAs without the use of radioisotopes.

In our study on the isolation of differentially expressed full-length cDNAs from a pair of near isogenic lines of mungbean, it was found that cDNA bandings generated by the CapFinder Kit were directly visualized on an agarose gel. No radioactivity was required to estimate yield or size distribution. It was also found that the differentially expressed cDNAs from the near isogenic lines were resolved nicely on the agarose gel. As a result, some differentially expressed cDNA species were recovered from the agarose gel and subcloned. The individual cloned cDNA showed positive results in Northern blot analysis, and their nucleotide sequences were determined. This method seems particularly useful in the isolation of differentially expressed abundant genes in a short period of time.

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Materials and Methods

Plant Varieties

Vigna radiata L. VC 1973A and VC 6089A were bred in the Asian Vegetable Research and Development Center (AVRDC). VC 6089A (BC_6S_2) populations were crossed using a wild mungbean TC 1966 as donor parents and breeding lines VC 1973A as recurrent parents.

RNA Preparation

Total RNA was extracted from the 15 DAF developing seeds of VC 1973A and VC 6089A by hot phenol method (Verwocrd et al., 1989). DNA-free total RNA was prepared as described previously (Oh et al., 1995).

Preparation of Full-Length Double-Stranded cDNA

The total RNAs were used to synthesize first and second-strand cDNAs using a CapFinder™ PCR cDNA library construction kit (Clontech). For the first step of the procedure, a 10 μl reverse transcription reaction mixture contained 1 × first-strand buffer, 10 μg of DNA-free total RNA, 1 μM of CapSwitch oligonucleotide (5'-TGCTGCGGAAGACGACAGAAGGG-3'), 1 μM of

CDS/3' PCR primer (5'-AATTCGAGCGGCCGCT$_{30}$VN-3', N= A, G, C, or T; V= A, G, or C), 1 mM of dNTP, 2 mM of DTT, and 100 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. The reaction was performed at 42°C for 1 h in an air incubator, and the reaction mixture was put on ice to terminate first-strand synthesis. For the second step (cDNA amplification by long-distance PCR), a 100 µl reaction mixture contained 2 µl of first-strand cDNA solution from the previous step, 1 × KlenTaq PCR reaction buffer, 0.2 µM of 5' PCR primer (5'-TGCTGCGGAAGACGACAGAA-3'), 0.2 µM of CDS/3' PCR primer, 200 µM of dNTP, and 1 × Advantage KlenTaq Polymerase mix. The mixture was heat-denatured in a GeneAmp PCR System 2400 (Perkin Elmer) at 95°C for 1 min and then amplified for 20 cycles (95°C for 15 sec and 68°C for 5 min). Aliquots of a 5 µl sample of the PCR product were run on a 1.5% agarose/EtBr gel. All of the different cDNA bandings at the 1.1–1.9 kb position was generated from differential expression between VC 1973A and VC 6089A. The major cDNA banding in the range of 1.1–1.9 kb was specific to VC 6089A, and entire various lengths of cDNAs were excised under long wavelength ultraviolet (UV) light and eluted from home-made spin column (Chuang and Blattner, 1994), followed by phenol-chloroform (1:1) extraction and alcohol precipitation. Northern blot analysis was performed at this stage to confirm these various lengths of cDNA mixture being specific to VC 6089A.

Northern Blot Analysis

The above mentioned cDNA mixture specific to VC 6089A was purified from agarose gel and used as a probe for Northern blot analysis as follows: 10 µg of Total RNAs purified from 15 DAF seeds of VC 1973A and VC 6089A were electrophoresed in a 1% agarose-0.41 M formaldehyde gel and blotted onto Hybond-N$^+$ membranes (Amersham) with Chomczynski's alkaline downward transfer method (Chomczynski, 1992). The membranes were incubated in 4 ml prehybridization buffer (Church and Gilbert, 1984) containing 7% SDS, 0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% albumin, and 2.5 mg/ml denatured salmon sperm single-strand DNA for 5 h at 65°C and then in hybridization buffer (newly prepared prehybridization buffer) containing a random-primed [^{32}P] cDNA probe (1 × 10^6 cpm/ml) (Feinberg and Vogelstein, 1984). Hybridization was performed at 65°C for 16–24 h. The membranes were washed twice with 2 × SSC, 1% SDS and 0.2 × SSC, 1% SDS for 15 min at 65°C. Signals were visualized and analyzed using a PhosphorImager system (Molecular Dynamics). The blots were striped and rehybridized with a probe specific to 25S ribosomal RNA to normalize them for equivalent loading of total RNA.

Cloning and Sequencing of Differentially Expressed cDNAs

These above mentioned various lengths of cDNA mixture that showed positive results in Northern blot analysis were ligated with pGEM-T easy vector (Promega) for 16 h at 14°C. After transformation using a standard $CaCl_2$ method (Sambrook et al., 1989) with the host strain DH5α, one-fifth of the transformation mixture was directly spread onto MacConkey-lactose agar plate (Jennings and Beacham, 1989) (Difco) supplemented with 100 µg/ml of ampicillin (without addition of isopropyl-β-D-thiogalactopyranoside). The plates were incubated overnight at 37°C. White recombinants were easily distinguishable from red non-recombinants. The white colonies were randomly picked, and insert length was checked by colony PCR. We selected ten clones based on various lengths of insert ranging from 1.1 to 1.9 kb. Each cDNA clone was used as a probe for further Northern blot analysis and sequencing. Nucleotide sequences were determined by directly sequencing double-stranded DNA templates using Sanger's dideoxy sequence method (Applied Biosystems Taq DyeDeoxy Terminator Cycle Sequencing Kit), and the homology search was carried out using the FastA program of the Wisconsin Package version 9.0 (Genetics Computer Group, Madison, Wisc).

Results and Discussion

Figure 1A shows agarose gel electrophoresis of double stranded cDNA synthesized with the CapFinder Kit using 10 µg of total RNA purified from the 15 DAF seeds of two mungbean varieties VC 1973A or VC 6089A. The differentially expressed cDNAs in the range of 1.1–1.9 kb generated from these two varieties were visible directly on the gel. These various lengths of cDNA mixture on the gel were purified as described under Materials and Methods and used as a probe for Northern blot analysis. Positive results were obtained (Figure 1B). Mungbean VC 1973A and VC 6089A are a pair of near isogenic lines with an approximate 1% sequence difference between both genomes (470–560 × 10^6 bp) (Young et al., 1992). These 1% sequence differences of genome could be potentially transcribed to many differentially expressed RNAs. The CapFinder Kit was based on a PCR technique which would probably preferentially amplify PCR products with sizes less than 2 kb DNA. Therefore, we observed these major DNA bandings in the range of 1.1–1.9 kb on the agarose gel.

The above mentioned cDNA mixture was subcloned and sequenced. All ten cDNA clones, CF2 (ca. 1800 bp), CF4 (1431 bp), CF22 (ca. 1700 bp), CF26 (ca. 1800 bp), CF29 (ca. 1800 bp), CF34 (ca. 1900 bp), CF41 (ca. 1900 bp), CF42 (ca. 1700 bp), CF69 (ca. 1200 bp) and CF71 (ca. 1700 bp), showed positive Northern blotting (Figure 2). Database searches revealed that the deduced partial sequences of CF2, CF4, CF22, CF42 and CF69 gene products share similarity with unknown seed protein (X13211), dehydration-responsive protein RD22 precursor (Q08298), 1L-myo-inositol-1-phosphate synthase (U30250), phaseolin (U01121) and soybean basic 7S globulin (X16469), respectively. The remaining clones, CF26, CF 29, CF34, CF41 and CF71 may be novel sequences as they have no homologous plant sequences in GenBank. Partial nucleotide sequencing indicated that six of the clones, CF2,

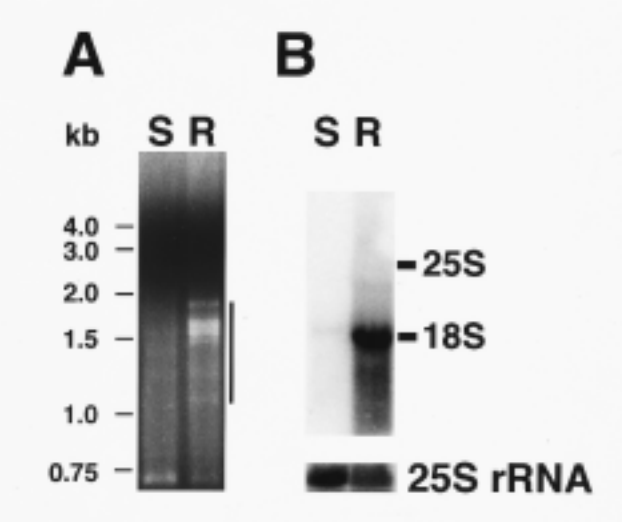

Figure 1. Agarose gel electrophoresis of double stranded cDNA synthesized with the CapFinder Kit using 10 μg of total RNA purified from the 15 DAF seeds of two mungbean varieties VC 1973A or VC 6089A. The vertical line indicates the different cDNA banding within 1.1–1.9 kb generated from these two varieties (A). These various lengths of cDNA mixture were purified and used as probes, which showed closely positive results in Northern blot analysis (B). S and R stand for for VC 1973A and VC 6089A mungbean, respectively. The blots were striped and rehybridized with a probe specific for 25S ribosomal RNA to normalize them for equivalent loading of total RNA. 25S and 18S stand for 25S ribosomal RNA and 18S ribosomal RNA, respectively.

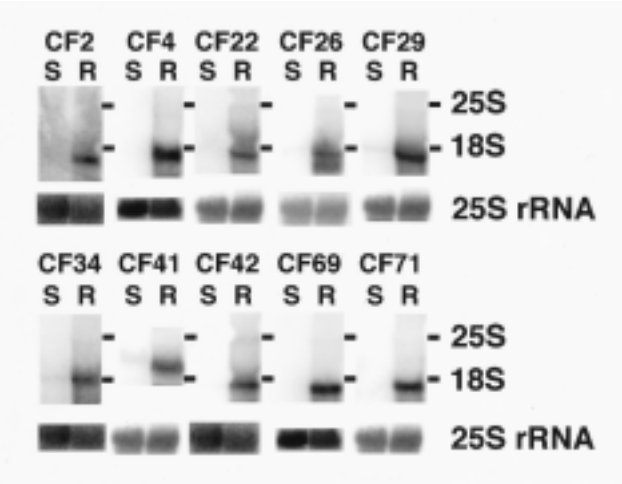

Figure 2. Northern blot analysis of ten cDNA clones that have various inserts ranging from 1.1 to 1.9 kb was performed using CF2 (ca. 1800 bp), CF4 (1431 bp), CF22 (ca. 1700 bp), CF26 (ca. 1800 bp), CF29 (ca. 1800 bp), CF34 (ca. 1900 bp), CF41 (ca. 1900 bp), CF42 (ca. 1700 bp), CF69 (ca. 1200 bp) and CF71 (ca. 1700 bp) as probes. Each blot was hybridized with a radioactive labeled probe as indicated cDNA clone. All cDNA clones showed positive results in the individual analysis. The blots were striped and rehybridized with a probe specific to 25S ribosomal RNA to normalize them for equivalent loading of total RNA. S and R stand for VC 1973A and VC 6089A mungbean, respectively.

CF4, CF22, CF41, CF42 and CF69, have a 5'-untranslated region and poly A^+ tail. Because total RNAs were transcribed to first-strand cDNAs with a CDS/3' PCR primer (5'-AATTCGAGCGGCCGCT$_{30}$VN-3', N = A, G, C, or T; V = A, G, or C) not a common oligo dT$_{12\text{-}18}$ primer, these six clones (CF2, CF4, CF22, CF41, CF42 and CF69) were probably full-length cDNAs (Figure 3). In the conventional method of cDNA library construction, only trace amounts of double-stranded cDNAs were synthesized. The estimation of yield or size distribution therefore requires radioisotopes. The method described in this paper is a PCR-based technique for making a high yield of cDNA libraries from only 50 ng or more of total RNA (Zhu et al., 1996). No radioactivity is required to estimate yield or size distribution, since it was found that cDNA bandings generated by this method were directly visualized on an agarose gel.

CF 2
GGTATCAGCTTCTCAGCATTAACCACTTTGCTTCTGCTATGGAGTTTCAG
TGCCTTGCATTGTTTTTTTCTCTCATTGTGATACTGATGGCTGCTCAGGC
TTCCTTACCTTCAGAAGTTTACTGGGAAAGGAAGCTTCCAAACACACCAA
TCCC·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CF 4
GATCAGCTTCTCAGCATTAACCACTTTGCTTCTGCTATGGAGTTTCAGTG
CCTTGCATTGTTTTTCTCTCTCATTGTGATACTGATGGCTGCTCAGGCTG
CCTTACCTCCAGAAGTTTACTGGGAAAGGATGCTTCCAAACACACCAATC
CCCAAAGTAATCAGACAATTTTCCGAACTAGATGGTGGCCAAGAGATTGC
ATCA·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CF 22
GATACAAATCCTTCTTTCTTCTCGGTGTACTGCACAGGGCTACCTCTTTG
TTTCATTATTTTTCATATTTTCTTCTTCTTCGTCTTCCATTCTTAATATC
TGATAAAAATGTTCATTGAGAATTTTAAGGTTGAGAGTCCTAATGTGAGG
TACA·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CF 41
GGATGAGGATTAGGATGAGGATGAAGAAGAAAAGCCTCGCCGCACTCGGG
GACCAACCCCCAGCCCCAAAGGAGAAGGACACACAGGGGTGGAAGTTGAG
GAAGATGAATCTCATGAGTCCGTGCGACACAAGACCCGCCACGAAGAGAG
TTTG·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CF 42
GATCATTCAGAGTCATCCATCAGAGTTTTGTATTACAACTCCGCCCATAT
TCACTAATACCATACTATTGTGAGAGCAAGGATTCCACTGCTGCTGTTGC
TGGGAATTCTTTTCCTGGCATCACTTTCTGTCTCCTTCGGCATCGTAGAC
CGGGGGAACATTGATGGCGCAGAAATGTCCGTTTCAAGAGGAAAAAATTA
CCCT·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CF 69
TGCTTGTTCTACCTACTCAAAAACATGCTTCCACTGGTCTCCATTGGACC
AACCTTCTCAAAAGAACACCTCTAACACAAGTACCAGTCCTAGTGGACCT
CAATGGACACCAGGTGTGGCTCAACTGTGAGCAACATTACTCATCCAAAA
CCTA·····AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3. Partial nucleotide sequences of clones CF 2, CF 4, CF 22, CF 41, CF 42 and CF 69, which were differentially expressed cDNA clones and isolated from the 15 DAF developing seeds of VC 6089A. The solid underlines indicate 5'-untranslate regions and poly A^+ tails of nucleotide sequences. The complete nucleotide sequence of CF 4 (1431 bp) was determined. The sequences of the other five clones were only partially determined. The number of base pairs determined for each clone was indicated in parenthesis: CF 2 (500 bp), CF 22 (500 bp), CF 41 (500 bp), CF 42 (500 bp) and CF 69 (500 bp).

In our differential display experiments using the CapFinder Kit, a number of cDNA clones were obtained. All of them showed positive Northern blotting. In addition, none of these clones were false positive because the high yield of differentially expressed cDNAs were purified from agarose gel and ligated directly with pGEM-T easy vector, thus avoiding further PCR amplification used in the RNA fingerprint by an arbitrarily prime PCR or differential display of RNA. The above-mentined PCR amplification step might cause a high rate of false positive results. With the several advantages described above, this method is obviously useful in the isolation of abundant, differentially expressed RNA.

In summary, the method described in this paper is well suited for use in the early stage studies of differentially expressed genes, especially for the purpose of isolating expressed abundant genes, because potentially full-length cDNAs can be obtained within a short period of time. This method would also be useful in the studies of stress response and gene induction in biological systems.

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