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Publish by Abstract

mRNA Differential Display Visualized by Silver Staining Tested on Gene Expression in Resurrection Plant *Boea hygrometrica*

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Abstract. A new protocol for nonradioactive mRNA differential display with high sensitivity visualized by DNA silver staining is presented. The protocol has been tested on total RNA of resurrection plant *Boea hygrometrica* to identify genes differentially expressed in dehydrated leaves compared to fresh and re-hydrated leaves. Total RNAs were reversely transcribed using three anchored oligo-dT primers. PCR amplification of relative cDNAs was carried out in combination with eight arbitrary primers. Urea-denatured polyacrylamide gel electrophoresis is used to separate amplified products of various molecular weights. Bands of amplified transcripts were resolved by silver staining, and the sensitivity was high enough to detect the tiny differences in DNA electrophoresis pattern. Several specific transcripts were successfully identified by this procedure, and one of them was cloned and sequenced.

Key words: *Boea hygrometrica*, DNA cloning, mRNA differential display, resurrection, silver staining

Abbreviations: RWC, relative water content.

Introduction

Poikilohydric or resurrection plants, found in many different families, are so called because of their unique ability to survive extreme desiccation for a long time and recover soon after re-hydration (Walter, 1955; Galf, 1974). This character can be studied in order to identify mechanisms of desiccation tolerance. Observations based on many years indicate that *Boea hygrometrica*, grown in China, is a resurrection plant. Its leaves are still viable when they are dried to 1% relative water content, and they recover after re-hydration. Photosynthesis is disturbed by desiccation but recovers by resurrection (un-

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published data). One approach to finding the mechanism of survival is to investigate gene expression in desiccated and re-hydrated leaves.

Though much progress has been made by cDNA library and hybridization screening and in vitro translation in the resurrection plant *Craterostigma plantagineum* (Bartels et al., 1990; Ingram and Bartels, 1996), the newly developed protocol of mRNA differential display permits the differential gene expression pattern to be investigated (Liang and Pardee, 1992). Until now, radioautography was used mainly for the detection of amplified transcripts. However, radioautography is time-consuming and sometimes problematic. For example, it is not feasible in every laboratory and more importantly, recovering a unique radiolabeled DNA species from the dried polyacrylamide gel is technically challenging, and often results in the failure to isolate and clone a particular cDNA (Lohmann et al., 1995). Alternatives to this method are under investigation (Boschi and Vergara, 1998; Lear et al., 1995). The detection of fluorescent labels requires expensive equipment or a complex operation. Ethidium bromide staining lacks of sensitivity itself (Sambrook, 1989). One possible way to visualize the amplified transcripts of mRNA is by silver staining. In this commentary we present the protocol for mRNA differential display detected by silver staining. The method proved to be inexpensive, simple and practical. To our knowledge, this is the first report of silver staining mRNA differential display tested on plant material.

Materials and Methods

Equipment

Rapidcycler (Idaho Technology) Thermal Cycler
Mini-PROTEAN Electrophoresis Cell (Bio-Rad)
377 DNA Sequencer (Perkin-Elmer)
Agarose Electrophoresis Equipment

RNA extraction

TNP buffer (0.42 M triisopropylnaphthalene sulfuric acid Na-salt (SERVA), 4.2 mM 4-aminosalicylic acid Na-salt (SERVA), 0.35 M Tris buffer, pH 8.0)
phenol: chloroform: isoamyl alcohol (50:49:1,v/v)
0.2 M Sodium acetate (pH 5.2)

mRNA differential display

Anchored primers ($HT_{11}M$: 5′-AAGCTTTTTTTTTTTA/G/C-3′): synthesized using Oligo DNA synthesizer (Beckman)
Arbitrary primers (HAP25: 5′-AAGCTTTCCTGGA-3′; HAP26: 5′-AAGCTT-GCCATGG-3′; HAP27: 5′-AAGCTTCTFCTGG-3′; HAP28: 5′-AAGCTTAC-GATGC-3′; HAP29: 5′-AAGCTTAGCAG-CA-3′; HAP30: 5′-AAGCTTCGT-ACGT-3′; HAP31: 5′-AAGCTTGGTGAAC-3′; HAP32: 5′-AAGCT -TCC TGCAA-3′): synthesized using Oligo DNA synthesizer(Beckman)
RNA PCR kit (Perkin-Elmer)

PAGE

Acrylamide (Bio-Rad)
N'N'-bis-methylene-acrylamide (Bio-Rad)
Urea (Promega)
10 × TBE buffer (For 1 l, 108 g of Trizma base, 55 g of boric acid and 3.7 g of EDTA)
10% ammonium persulfate
TEMED (Sino-American Biotechnology Company)
Loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.01% bomophenol blue)

Silver Staining

7.5% acetic acid (v/v)
Silver Stain kit (Bio-Rad)

Cloning and Sequencing

Hind III and its buffer (GIBCOBRL)
Agarose
Magic PCR Preps^{TM} DNA Purification System for Rapid Purification of DNA Fragments (Promega)
pUC19 vector (Sino-American Biotechnology Company)
T_4DNA ligase (Promega)
E. coli JM109 (Sino-American Biotechnology Company)
M13(-20)Forward primer

Plant Material

Plants of *Boea hygrometrica* collected from their natural environment in the suburb of Beijing City were grown under normal conditions. Fresh leaves, dehydrated leaves (3 or 24 hr in 50% air RWC) and re-hydrated leaves (100% air RWC after dehydration) were frozen in liquid nitrogen before the extraction of total RNA.

Total RNA Extraction

Total RNA was extracted from leaves as described by Burgermeister et al. (1986). 0.5 g leaves from several plants, treated equally, were pooled for one isolation after purification by phenol: chloroform: isoamyl alcohol (50:49:1, v/v) and ethanol precipitation. RNA was re-suspended in a total volume of 100 μl ddH_2O. Agarose gel electrophoresis was used to test the integrity and purity of the RNA extracted.

mRNA Differential Display

mRNA differential display was carried out as described in the handbook of GenHunter Corporation (1994). Total RNA was used for reverse transcription. For each RNA sample, three reverse transcription reactions were set up, each containing one of the three different $HT_{11}M$ primer (where M may be G, A or C) anchored to the beginning of the poly(A^+)tail. MMLV reverse transcriptase was added after pre-incubation at 65 ° for 5 min and at 37 ° for 10 min. Reverse transcription was performed by incubation at 37 ° for 50 min, then was stopped by inactivating the MMLV reverse transcriptase at 75 ° for 5 min. The products of reverse transcriptions were stored at 4 ° for recent use or at −20° for later use.

PCR amplification of each reverse transcription products was carried out in combination with one of eight arbitrary primers. PCR was carried out as follows: 94° for 30 s; 40° for 2 min; 72° for 30 s for 40 cycles and finally 72° for 5 min.

The amplified cDNA subpopulations of 3′-termini of mRNAs as defined by this pair of primers were size fractionated by denaturing polyacrylamide electrophoresis using Bio-Rad Mini-PROTEAN Electrophoresis Cell. 3.5 μl of each PCR sample were incubated with 2 μl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.01% bromophenol blue) at 80° for 2 min immediately before loading.

Silver Staining

Silver Staining of the polyacrylamide gel was performed as described by the Bio-Rad Silver Stain Handbook, with the modification that the gel was fixed using 7.5% acetic acid (v/v) immediately after electrophoresis and the developmental reaction was stopped using 7.5% acetic acid (v/v).

Cloning and Sequencing of the Differentially Expressed cDNA Fragment

The gel band of differentially expressed cDNA fragments was cut down with a knife and added to an Enppendorf tube to boil for 15 min. The extract served as the template to re-amplify with the same pair of primes as in PCR. Since all the primers contained built-in restriction sites of *Hind* III, the product of re-amplification was firstly digested with *Hind* III (Promega), run on a 2% agarose gel to remove short oligonucleotides and residual dNTP and then the proper band was cut down and cleaned by Magic PCR Preps^{TM} DNA Purification System for Rapid Purification of DNA Fragments (Promega), and finally, the purified digested DNA was directly cloned into pUC19 by T_4DNA ligase, then transformed into *E. coli* JM109. It was sequenced with the M13 (−20) Forward primer using 377 DNA Sequencer (Perkin–Elmer).

Result and Discussion

Amplified transcripts of RNA extracted from the differently treated leaves were clearly visualized by silver staining after separation by PAGE (Figure 1). For every combination of the three anchored primers and eight arbitrary primers, numerous identical bands of amplified cDNA were displayed.

The majority of these bands were present in nearly the same amount in all leaves though some of them were up-regulated during dehydration (Figure 1D). Quite a few showed differential expression patterns. Some bands (Figure 1B and Figure 1C) existed in fresh leaves but disappeared after dehydration and recovered after re-hydration. Some existed only after re-hydration (Figure 1E). And for seven pairs of primers, ten bands existed only in dehydrated leaves, Figure 1A, which were identified as the transcripts induced by dehydration. One of them amplified with $HT_{11}C$ and HAP26 (Figure 1A) was re-amplified with the same pair of primers and cloned into pUC19 vector. The sequence data shown in Figure 2 support that it is mRNA amplified product, since its 3'-termini sequence with a poly(T) tail is similar to the anchored primer of $HT_{11}C$, and its 5'-termini sequence is complementary to the arbitrary primer of HAP26.

The above results suggest that the silver staining method is feasible and sensitive enough to visualize the amplified products of mRNA differential

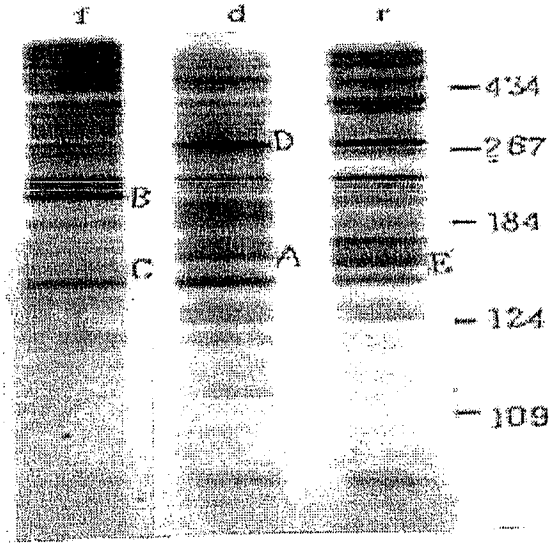

Figure 1. mRNA differential display visualized by silver staining (primers were $HT_{11}C$ and HAP26). Line f, d and r represent DNA fragments amplified from fresh, dehydrated and re-hydrated leaves relatively. (A) cDNAs expressed only in desiccated leaves; (B) cDNAs disappeared during dehydration; (C) cDNAs expressed only in fresh leaves; (D) cDNAs up-regulated in desiccated leaves; (E) cDNAs expressed only in re-hydrated leaves.

5'-AAGCTTTTTTTTTTCCATTGTCGTATCCTTCGCTTGCCGTGTCTTGTCCATAGTC

GCGTCCTTGGCTTCACCGGCTTTCTGCATCGTCGAATCCTTAGCTTGCTGCGATTTGT

CCACAGCATAGTCTCTGTACTCTCCAATTTTCCCCTCCATGGCAAGCTT-3'

Figure 2. Sequence data of the re-amplified cDNA fragment amplified by the primers of $HT_{11}C$ and HAP26.

display. The same result was obtained by Lohmann et al. (1995). They compared radioactive and silver staining methods for detection of the display of hydra cell mRNAs and proved that the latter was sensitive enough to detect low abundance mRNA (less than 1 ng of DNA). They stated that the most important factor was 'the probability of recovering a band of interest, and thus its successful re-amplification, is increased considerably compared to the standard radioactive procedure because of the visibility of the cDNA band in the original gel'. Because of its simplicity and timesaving ability, silver staining has been successfully used to detect the products of RAPD (Hu et al., 1997), DNA amplified fingerprinting (Caetano-Anolles and Bassam, 1993) and DNA sequencing (Promega), and it may also promote the further use of mRNA differential display.

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