Plant Molecular Biology Reporter **20:** 231–238, September 2002

Genetic Resources

Suppression Subtractive Hybridization Cloning of cDNAs of Differentially Expressed Genes in Dovetree (*Davidia involucrata*) Bracts

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Abstract. A subtracted cDNA library for *Davidia involucrata* was constructed using suppression subtractive hybridization (SSH). mRNA isolated from young leaves was used as a "driver," and mRNAs isolated from young bracts were used as "testers." The differentially expressed cDNA fragments in bracts were identified by differential screening. Of the 16 clones selected randomly from the screened library, 8 were known genes found in GenBank, and 2 had no similar sequences. Northern blot analysis revealed that the expression level of P1A5 cDNAs selected randomly was dominantly expressed in bracts. This indicates that SSH can be used to clone differentially expressed cDNAs in *D. involucrata* bracts.

Key words: bract, *Davidia involucrata*, leaf, northern blot analysis, SSH

Abbreviations: DIG, digoxigenin; SSH, suppression subtractive hybridization.

Introduction

Davidia involucrata Baill., an ornamental tree known as the Chinese dovetree or handkerchief tree, is a relic deciduous tree species of the Tertiary period. It is the only species in the family Davidiaceae. Presently, the Chinese dovetree survives only in Southwest China, although it used to be more widespread (Browne and McDonald, 1978; Wyman, 1978; McClintock, 1991; Zhang et al., 1995). The identifying feature of the Chinese dovetree is a hanging flower cluster of 2-3 large white bracts surrounding an inflorescent head. The bracts are generally 3-6 inches long. Surprisingly, since the species' discovery in 1869 (McClintock, 1991), no research on the function of bracts in evolutionary and reproduction biology or on their developmental mechanisms has occurred.

We investigated the developmental mechanism of dovetree bracts. Using bracts as testers and leafs as drivers, we constructed a subtracted library using suppression subtractive hybridization (SSH) (Diatchenko et al., 1996). Dot

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blotting and northern blotting were used to identify those cDNAs specifically expressed or overexpressed in bracts.

Materials and Methods

Materials

Young leaves and bracts of *D. involucrata* were collected immediately after they expanded (<2 cm in length) at the end of March and the beginning of April 2001. Harvested materials were promptly rinsed with distilled water, dried with filter paper, frozen with liquid nitrogen for several minutes, and stored at -80ºC.

RNA isolation

Total RNA was extracted from frozen leaves and bracts using the Watson kit of total RNA isolation for plant leaves (Watson, China). To perform SSH, total RNA was pooled for both leaves and bracts. Poly $(A)^+$ RNA was purified with the RNeasy Mini Kit (QIAGEN).

Suppressive subtractive hybridization

SSH was performed using the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Double-stranded cDNA was prepared from 2 µg of poly $(A)^+$ bract RNA (tester population) and leaf RNA (driver population). The cDNA was then digested with *Rsa* I. In 2 separate ligations, tester cDNA was ligated to adapters 1 and 2. In the first hybridization, an excess of driver cDNA was hybridized at 68ºC for 8 h with tester cDNA ligated to adapter 2 in reaction 2. In the second hybridization, reactions 1 and 2 were hybridized together in the presence of fresh driver cDNA at 68ºC overnight. The subtractive product was amplified by PCR using oligonucleotides that were complementary to adapters 1 and 2. PCR was performed according to the following parameters: 75ºC for 5 min and 27 cycles at 94ºC for 30s, 66ºC for 30s, and 72ºC for 1.5 min. Then, a nested PCR was performed as follows: 12 cycles at 94ºC for 30s, 66ºC for 30s, and 72ºC for 1.5 min. The final PCR product was identified as dominantly expressed cDNA and corresponded to the gene population overexpressed in the bracts. In the same manner, we performed the reverse-subtractive hybridization to obtain genes repressed in the bracts. This final product was identified as dominantly expressed cDNA in leaves.

Subtracted library construction

cDNA dominantly or specifically expressed in bracts was purified using QIAquick PCR Purification kit (QIAGEN) and cloned into a pMD 18-T vector [TaKaRa Biotechnology (Dalian) Co., China]. Colonies were grown on LB agar plates containing ampicillin (Kodak, New Haven, CT), X-Gal (Gibco BRL), and IPTG (Gibco BRL). Transformation efficiency was approximately 10^7 colonies per µg of starting DNA.

Screening of the subtractive clones

A total of 250 transformants were randomly selected from the subtracted library to amplify the inserted sequences. Each reaction tube contained 2.5 µL 10X *Ex Taq* Buffer, 2 µL MgCl (25 mM), 2 µL dNTP (2.5 mM each), 1 µL of nested primer 1 and nested primer 2R (10 µM), 16.375 µL of PCR-grade water, and 0.125 µL TaKaRa *Ex Taq*. PCR was performed according to the following parameters: 95ºC for 30s and 25 cycles at 95ºC for 10s and 68ºC for 2 min. PCR products were analyzed by electrophoresis on 1.2% agarose gel. We selected 237 positive clones on which to perform dot-blotting analysis. We mixed 5 µL PCR product of each positive clone with 5 µL 0.6N NaOH. Then, 1 µL of mixture was applied to a positively charged nylon membrane (Gene Company Limited). Two membranes with the same lattice pattern were obtained and baked under a vacuum at 80ºC for 2 h. The 2 membranes corresponded to 2 probes: DIG-labeled forward-subtracted and reverse-subtracted products. We labeled 15 µL of the second PCR products with digoxigenin-dUTP according to the manufacturer's instructions using the DIG High Prime DNA Labeling and Detection Starter kit II (Roche Molecular Biochemicals). The labeled probes were blocked out by adding a blocking solution (10 mg/mL salmon sperm DNA, 0.3 mg/mL oligonucleotides complementary to the nested primer 1, and 2R). The hybridization and detection were performed according to the manufacturer's instructions. The membranes were prehybridized for 30 min and then hybridized with denatured DIG-labeled probes for 12 h. The membranes were washed with 0.5 SSC and 0.1% SDS. After being incubated in blocking solution, antibody solution, washing solution, and detection solution, progressively, the membranes were subjected to immunological detection. We applied ready-to-use CSPD to the membranes and exposed them to x-ray film at 20ºC for 15-20 min. Clones that had relatively strong signals when hybridized with a probe of positively subtracted cDNA were selected.

Northern blot analysis

Northern blot analysis was performed with selected potential clones dominantly expressed in bracts. The inserted sequence was amplified and labeled with DIG. mRNA from leaves and bracts was extracted, electrophoresed on a 1% formaldehyde gel, and downward transferred onto positively charged nylon membranes (Sambrook et al., 1989). The membranes were hybridized with labeled probes at 50ºC, washed as recommended (Roche Molecular Biochemicals), and exposed to Kodak x-ray film.

Sequencing and sequence analysis

The 16 selected positive clones dominantly expressed in bracts were sequenced with DYEnamic Direct dGTP Sequencing Kit (Amersham) and a 373A DNA sequencer. Sequence analysis was performed with the Blast program, DNATool 5.1, and OMIGA 2.0.

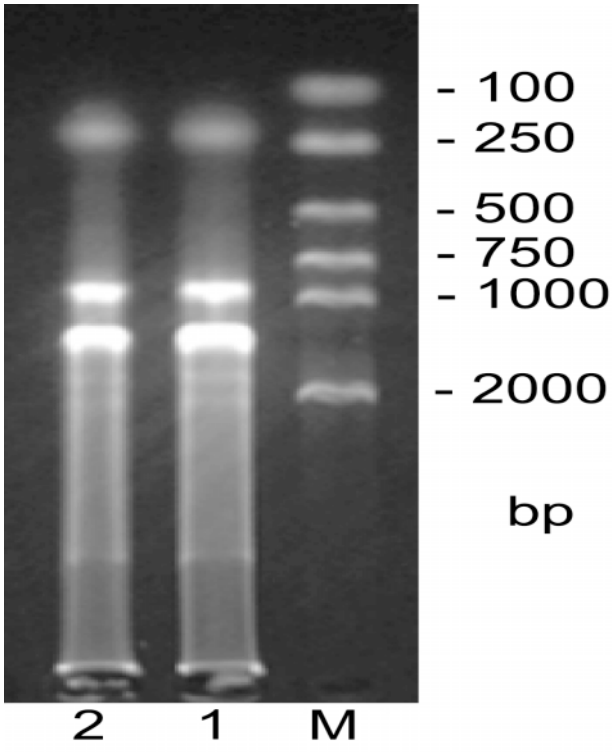

Figure 1. Total RNA of bracts (lane 1) and leaves (lane 2).

Results

Qualitative and quantitative analysis of RNA

Using the RNA extraction kit described above, total RNA was isolated from leaves and bracts during their early expanding stage. The RNA appears undegraded on 1% agarose gel (Figure 1a). Typical A_{260}/A_{280} absorbance ratios of the RNA range from 1.8-2, indicating that little or no protein contamination has occurred (Schultz et al., 1994). The A_{260}/A_{230} ratios are greater than 1, indicating that little or no polysaccharide or polyphenol contamination exists (Schultz et al., 1994). Yields are in the range 0.5-1.8 mg/g fresh weight. The A_{260}/A_{280} ratios of purified poly $(A)^+$ RNA of both materials are greater than 1.9. A clear smear greater than 0.5 kb is present on the 1% agarose gel, indicating that the quality of the obtained poly $(A)^+$ RNA is sufficient for further use.

Identification of subtraction efficiency

The key to obtaining successful SSH results was to effectively eliminate uniform cDNA appearing in both testers and drivers (Diatchenko et al., 1989). The *D. involucrata* 18S rRNA gene (GenBank Accession No. AJ235991) sequence was searched in GenBank and analyzed for restriction sites of *Rsa* I. Two *Rsa* I restriction sites are expressed in both leaves and bracts. Two primers, 18S5 and 18S3,

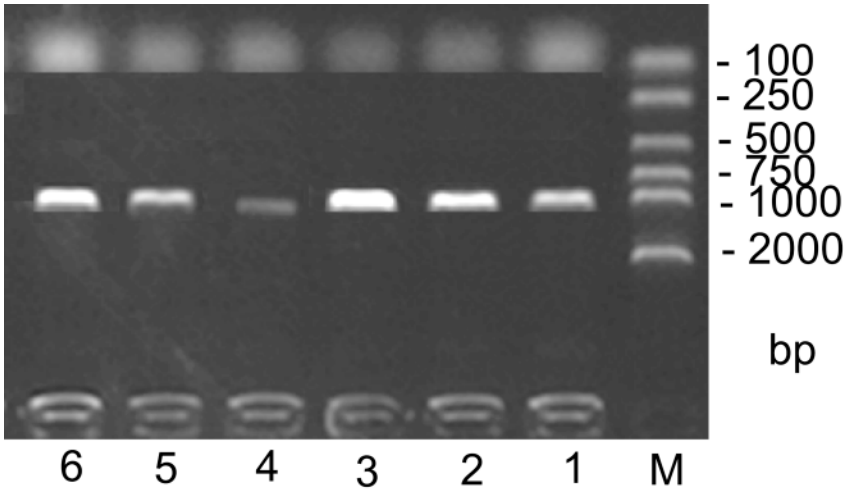

Figure 2. PCR analysis of subtraction efficiency. PCR was performed on subtracted (lanes 4-6) and unsubtracted (lanes 1-3) secondary PCR products with primers 18S5 and 18S3 of *D. involucrata* 18S rRNA gene. Lane 1, 22 cycles; lane 2, 27 cycles; lanes 3 and 4, 33 cycles; lane 5, 38 cycles; lane 6, 40 cycles. M: DNA size markers.

were designed to amplify the longest segment (1116 bp) between the 2 *Rsa* I restriction sites. PCR amplification of this segment (Figure 2) shows that it appeared after 24 cycles when using the unsubtracted tester cDNA as a template but did not appear until after 33 cycles when using the subtracted cDNA as a template. This indicates that cDNA homologous to both tester and driver was eliminated. The SSH results are shown in Figure 3. Clearly, there are a few distinct bands between cDNA of subtracted and unsubtracted libraries. This indicates that differentially expressed genes are enriched by 2 rounds of suppressive PCR amplifications.

Identification of positive clones and differential screening

The second PCR products of SSH were cloned into pMD-18T vectors after purification and preserved in *Escherichia coli* JM109. Screening by blue-white spots indicated that approximately 95% of transformants contain inserts. Of the 250 clones randomly selected, 237 contain inserts (ranging from 100-2000 bp), as evidenced by PCR screening (Figure 4). All 237 clones were also screened by dot-blotting analysis (Figure 5). Finally, 167 clones potentially overexpressed in bracts were chosen. Among them, 16 clones were randomly selected to have their inserts sequenced (unpublished results). Sequence analysis of these 16 inserts revealed that 4 of them (P1B4, P1C4, P1D12, and P3E8) are the same insert (GenBank Accession No. AY059472); P1A5 and P2H2 are the same (GenBank Accession No. AY059473); and P1A11 and P1F4 are the same. The other 8 clones are different. Homologous comparison of these 16 inserts with GenBank by the Blast program indicated that 2 cDNA clones (P1D4 and P2E2) are new genes without any homology to the GenBank, and that the others are more or less

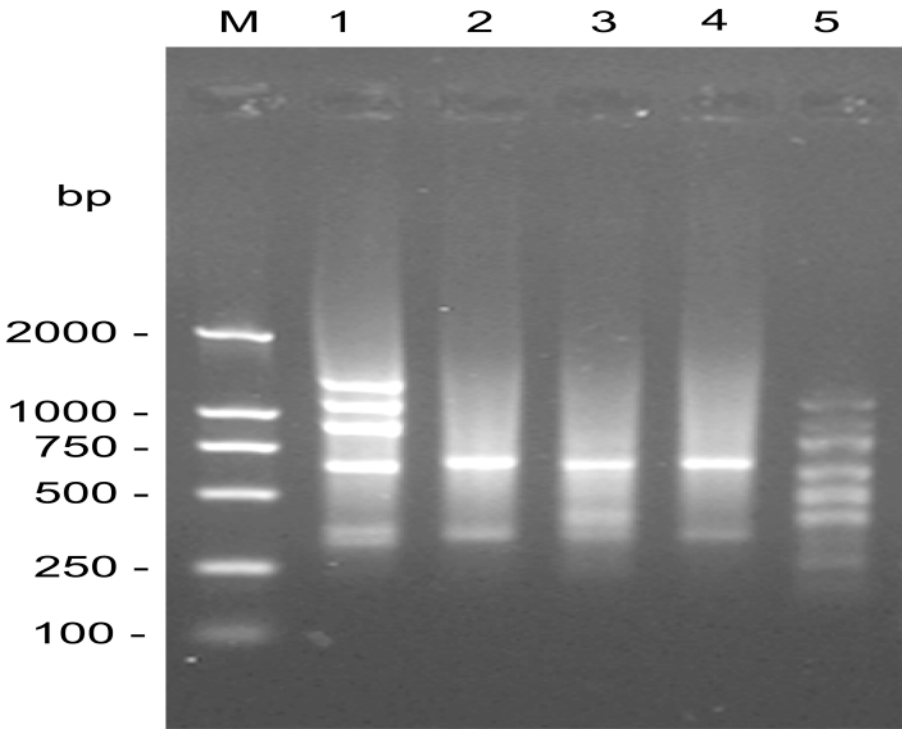

Figure 3. Results of SSH using bracts as testers and leaves as drivers. This shows the secondary PCR products. Lane 1, control; lane 2, unsubtracted leaf cDNA; lane 3, reverse-subtracted leaf cDNA; lane 4, unsubtracted bract cDNA; lane 5, forward-subtracted bract cDNA. M: DNA size marker.

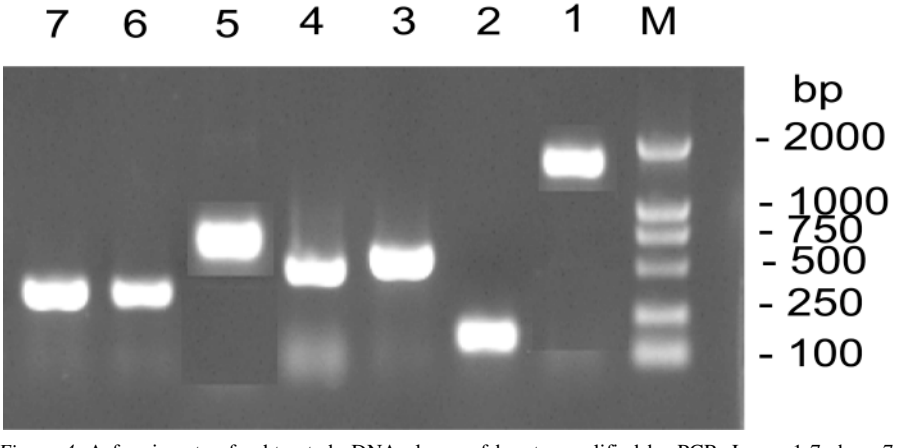

Figure 4. A few inserts of subtracted cDNA clones of bracts amplified by PCR. Lanes 1-7 show 7 clones randomly selected from the subtracted library. M: DNA size marker.

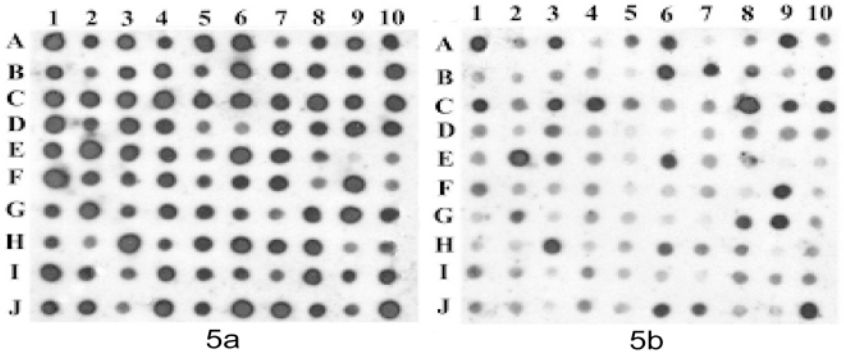

Figure 5. Differential screening of positive clones of the subtracted library. In all, 237 clones of subtracted bract cDNA were arrayed on positively charged nylon membranes and hybridized to different probes labeled by DIG (here only 100 clones are shown). 5a, hybridized to forward-subtracted bract cDNA probe; 5b, hybridized to reverse-subtracted leaf cDNA probe.

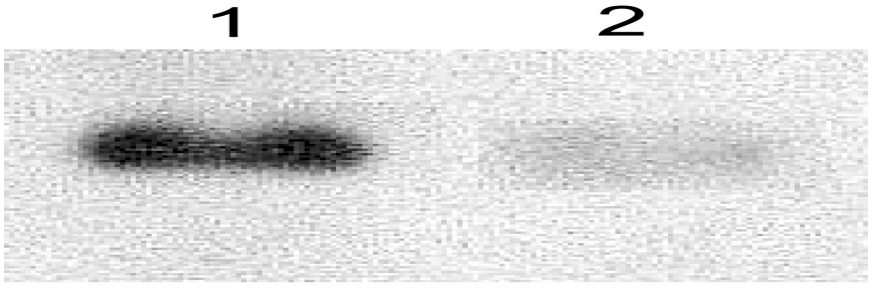

Figure 6. Northern hybridization of dovetree total RNA and probe of fragment spanning the coding region of P1A5 gene. Lane 1, RNAs from bracts; lane 2, RNAs from leaves.

homologous to known genes (e.g., P1D10 clone, GenBank Accession No. AF448811).

Northern blot analysis

The expression of the P1A5 gene (GenBank Accession No. AY059472) was examined in young bracts and leaves by northern blot analysis. We isolated 10 µg total RNA of bracts and leaves and examined it on a 1% agarose gel. A fragment spanning the coding region was amplified and labeled with nonradioactive digoxigenin-dUTP and was used as a probe in hybridizing equal amounts of total RNA from both tissues. A single RNA transcript (approximately 1000 bp) was detected in both bracts and leaves. However, the signal intensity in bracts (Figure 6, lane 1) is quite strong compared to that of leaves (Figure 6, lane 2). This indicates that the P1A5 clone is enriched in bracts by SSH.

Discussion

Involucrum (bract) is an important part of an inflorescence. However, mechanisms involved in the development of involucrum are not well understood. Bracts are thought to be modified leaves or have evolved from leaves (Ravan et al., 1992). In understanding their developmental mechanisms, comparing the developmental progresses of both leaves and bracts may be useful. *D. involucrata* has large bracts, making it a perfect species in which to investigate the developmental aspects of bracts. We constructed a subtracted library with expanding bracts as testers and leaves as drivers using SSH to identify genes specifically expressed or overexpressed in bracts.

Our studies show that in using SSH, differentially expressed genes of bracts were enriched while most homologous cDNAs were eliminated. A subtracted library of bracts was constructed, and differential screening and northern blot analysis revealed that the subtraction was effective. Of the 16 clones randomly selected from the screened library, 2 new and 8 known genes were identified, including a gene coding *D. involucrata* lipid transfer protein precursor (GenBank Accession No. AY059472), a gene coding *D. involucrata* raucaffricine-O-beta-D-glucosidase-like protein (GenBank Accession No. AY059473), a gene coding putative transcription factor (clone P1A11), and a gene coding extension (clone P1C11). This is the first investigation that uses gene cloning to study the mechanisms involved in involucrum development. Our next step is to screen the entire subtracted library to identify genes specifically expressed in bracts. We hope to seek out genes related to the developmental mechanisms of bracts.

Acknowledgments

This work was supported in part by grants from the Project No. 39770134 of the National Natural Science Foundation of China and from the Department of Education of Sichuan Province, China.

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