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Full Length Research Paper

Construction of full-length cDNA library of white flower *Salvia miltiorrhiza bge f.alba* root and partial EST sequence analysis

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In order to screen and isolate secondary metabolite biosynthesis related gene, we construct a cDNA library of white flower *Salvia miltiorrhiza bge. f.alba.* High quality of total RNA was successfully isolated from roots of white flower *S. miltiorrhiza* using modified CTAB method. Double strand cDNA was cloned into pDNR-LIB vector. The number of clones, recombinant rate and length of insert fragments were determined. Results showed that the capacity of the original library was 1.8×10^7 with a recombinant rate of 91% and the inserted cDNA fragments ranged from 0.5 to 2.0 kb. Partial cDNAs chosen by random were sequenced. After BLAST analysis of some cDNAs, their possible functions were predicted. It is found that most of these cDNAs were similar to homological genes of *Arabidopsis thaliana*, *Oryza sativa*, and other plants. Most of the genes were related to cell metabolism, stress resistance, cell growth and development, etc. More importantly, some key enzymes and factors involved in secondary metabolism of *S. miltiorrhiza*, such as EST fragments of phenylalanine ammonialyase (*SmPAL*1), chorismate synthase (*SmCHS*), 3-hydroxy-3-methylglutaryl CoA reductase (*SmHMGR*), 4-Coumarate-coenzyme A ligase (*Sm4CL*1) and *SmMYB*90, were found from this library. These results indicated that the library has enough capacity, high recombinant rate and long insert fragment. This study provided a base for further study on the structure and function of these cDNAs.

Key words: white flower *Salvia miltiorrhiza bge f.alba*, RNA isolation, cDNA library construction, EST sequence analysis.

INTRODUCTION

Salvia miltiorrhiza Bge is a well-known traditional Chinese herb and broadly planted in China. Its roots (called Dan-shen in Chinese) contain two groups of biologically active compounds, caffeicacid-derived phenolic acids and various tanshinones belonging to the group of diterpene quinines (Zhou et al., 2005). It is one of the most popular traditional herbal medicines in some Asian countries and has been used clinically for the treatment of various ailments such as cardiovascular, cerebrovascular, hyper-lipidemia, and acute ischemic stroke diseases (Zhou et al., 2005; Jiao et al., 2007). Pharmacologic studies revealed its secondary metabolites with various biological activities and protective effects against cerebral and heart ischemia-reperfusion, inhibitory activity against hepatic fibrosis and hepatoprotection, and antioxidant activity, antithrombosis activity, antihypertension activity, antivirus activity, antitumor activity and anti-ulcer activity (Jiang et al., 2005).

White flower *S. miltiorrhiza* grown only in Shandong province of China is a variety of *S. miltiorrhiza Bge*. The morphological difference between white flower *S. miltiorrhiza* and *S. miltiorrhiza* Bge is the color of flowers. The flower color of *S. miltiorrhiza bge* is purple, while that of *S. miltiorrhiza* is white. Except for morphological difference, white flower *S. miltiorrhiza* also has special pharmacological effect for treatment of thromboangiitisob-literans (Hao et al., 2006). Result by Cui et al. (2007) showed that the contents of water-soluble active com-ponents, protocatechuic aldehyde (PAH), danshensu (DSS), and protocatechuic acid (PA) and in *S. miltiorrhiza* ranges from 2.5 to 200 μg mL^{-1}. Studies showed that

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caffeic acid-derived phenolic acids contents in white flower *S. miltiorrhiza* is about two times higher than that in *S. miltiorrhiza bge* (Hao et al., 2006). Qi et al. (2004) also reported that the trace elements in contents of Fe, Mg, Mn in white flower *S. miltiorrhiza* were more than that in *S. miltiorrhiza bge*. Jiao et al. (2007) reported that *S. miltiorrhiza* root preparation could inhibit the proliferation and induce the apoptosis of human gastric cancer cells. These results indicated that white flower *S. miltiorrhiza* has higher pharmaceutical values. Recently, our group successfully produced seedlings of *S. miltiorrhiza* from stems and leaves culture (Sun et al., 2008).

S. miltiorrhiza bge has increasingly attracted the attention of research groups in recent years especially in biotechnological field. Cell and organ culture systems had been used to produce the useful secondary metabolites of *S. miltiorrhiza bge* (Yuan et al., 1990; Wang et al., 1998; Huang et al., 2000). Hairy roots and crown gall cultures of *S. miltiorrhiza* bge were established by infecting sterile seedlings with wild *Agrobacterium rhizogenes* or *A. tumefaciens* strains (Chen et al., 1997; Song et al., 1997). Many methods based on hairy roots or crown gall cultures were taken to enhance the production of tanshinones or caffeic acid derived metabolites, including Ag^+ elicitation, nutrient feeding, beta-aminobutyric acid induction, *in situ* adsorption, and semicontinuous operation (Zhang et al., 2004; Ge and Wu 2005; Yan et al., 2005). However, little is known about the control point and biochemical or genetic cross-talk within and between pathways which will facilitate the engineering of existing metabolic targets of *S. miltiorrhiza bge*. Applying molecular biology technology can help to understand the mechanism of secondarymetabolite biosynthesis of *S. miltiorrhiza bge.* For instance, genetic transformation is a powerful tool to study gene functions in plants and could help to understand the factors that control flux into specific routes of secondary metabolism. Moreover, the number of chromosome of *S. miltiorrhiza bge* is less (2n=14), so it is suitable for being as the model medicinal plant of molecular biology study.

In recent years, gene regulation of secondary metabolite biosynthesis in medicinal plant is the hot study point of Chinese traditional medicine. Genome-scale collections of full-length cDNA can especially be utilized as additional useful information in gene discovery and subsequent functional assay (Ling et al., 2007). Full-length cDNA library is a powerful tool for functional genomics and is widely used as physical resources for identifying genes. A full-length cDNA library should be an important resource for studying secondary metabolite biosynthesis genes of white flower *S. miltiorrhiza*. However, RNA isolation from white flower *S. miltiorrhiza* is more difficult than from other common plant because it is rich in secondary metabolite, such as phenolic compounds and amylase. So far, there is no report about RNA extraction and cDNA library construction of white flower *S. miltiorrhiza.* The aim of present study is to isolate high quality total RNA, construct a full-length cDNA library, and to isolate genes related to secondary metabolite biosynthesis in *S. miltiorrhiza*, which could pave the way for investigation the molecular mechanism of these metabolisms pathway. In addition, this study also makes a foundation for conservation of germplasm resource of white flower *S. miltiorrhiza.*

MATERIAL AND METHODS

Plant material

Roots were harvested from 2-year-old white flower *S. miltiorrhiza*. After harvesting, roots were immediately frozen in liquid nitrogen and stored at –80℃ until used.

Solutions and reagents

All the solutions were prepared with DEPC-treated and autoclaved MilliQ-water (Millipore). All nondisposable plastic material was treated with DEPC and autoclaved. Glass material and the mortar and pestle were treated for 4 h at 180℃. Creator SMART cDNA Library Construction Kit was from CLOTECH. mRNA isolation kit was from Amersham Pharmacia Biotech.

Isolation of mRNA

The QuickPrep mRNA purification kit was used for isolation of mRNA. The eluted mRNA in 0.75 ml DEPC H_2O was dried in a cold vacuum and directly used for cDNA synthesis without ethanol precipitation.

First-strand cDNA synthesis

First-strand cDNA was synthesized according to the protocol of Creator SMART cDNA Library Construction Kit (Clontech,USA): 1 µl mRNA sample (about 1 µg), SMART IV oligonucleotide and CDS III/3' PCR primer: (oligonucleotide: 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'; CDS III/3' PCR primer: 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}N_{-1}$N-3' (N =A,G,C,or T; N_{-1}=A,G,or C) were incubated at 72℃ for 2 min. 5× first strand buffer, DTT, dNTP mix and Power Script reverse transcriptase were incubated at 42℃ for 1 h.

Amplification of cDNA by long-distance polymerase chain reaction (LD-PCR)

Two microliter of the first-strand cDNA, deionized H_2O, advantage 2 PCR buffer, dNTP mix, 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'), CDS III/3' PCR primer and advantage 2 polymerase mix were added into a new pre-chilled 0.5 ml tube, then amplified by the following program: 95℃, 20 s; 24 cycles of 95℃ 5 s, 68℃ 6 min. Five microliters of the PCR products were analysed on 1.0 % agarose/EtBr gel. The concentration of the double strand (ds) cDNA was roughly estimated by compared with DNA marker.

Proteinase K Digestion, *Sfi I*digestion

Fifty microliter amplified ds cDNA and 2 µl proteinase K (20 µg/µL) were added into a 0.5 mL sterile EP tube and incubated at 45℃ for 20 min. PCR product purification was carried out according to the

protocol of Qiaquick PCR Purification Kit (Qiagen, Germany). Finally, 30 µL deionized H_2O (pH 8.0) was used to elute purified DNA. Deionized H_2O was added up to 79 µL.

The following components were combined in a fresh 0.5 mL tube for *SfiI* digestion: 79 µL cDNA, 10 µL 10× Sfi buffer, 10 µL *Sfi I* enzyme, 1 µL 100× BSA, and added water to 100 µL total volume. Tube was incubated at 50℃ for 2 h, and then added to 2 uL of 1% xylene cyanol dye to the tube and mixed well.

cDNA size fractionation

The cDNA size fractionation was carried out using CHROMA SPIN-400 columns according to the protocol of CHROMA SPIN-400. Sixteen fractions were collected in the separated tubes. Three micoliter of each fraction was taken to run 1% agarose/EtBr gel alongside DNA size marker at 150 V for 10 min. The peak fractions were determined by visualizing the intensity of the bands under UV; Four fractions containing suitable cDNA were put together into a clean 1.5 mL EP tube. Sodium acetate, glycogen and 95% ethanol (-20℃) were added and placed in -20℃ freezer overnight and then centrifuged. The supernatant was carefully removed, and the pellet was washed by 70% ethanol and vacuum-dried. Seven microliter deionized H_2O was added to resolve the pellet, which was then kept at -20℃.

Ligation cDNA to pDNR-LIB vector

The pDNR-LIB vector was used for cDNA construction. The following components were added in a fresh 0.5 mL tube: 1 µL pDNR-LIB, 10 × ligation buffer 0.5 µL, ATP (10 mM) 0.5 µL, T4 DNA ligase 0.5 µL, water added to 5 µL for ligation reaction; the tube was incubated at 16℃ overnight.

Transformation of recombinant plasmids into *Escherichia coli*

The ligation mixture with 100 µL thawed cells (*E. coli* DH10B) was transferred to a chilled cuvette. It was electroporated by discharging, and then immediately removed the cuvette from the chamber. The entire volume was transferred to the prelabeled polypropylene tubes containing 900 µL LB broth. The tube was incubated with shaking (225 rpm) for 1 h at 37℃. Fifty microliters of aliquot was spread on a pre-warmed 90 mm LB agar plate containing 30 µg/mL of chloramphenicol. Incubate the plate at 37℃ overnight.

Quality analysis of cDNA library

The number of colonies on each plate were counted and the titer calculated as described below. The library tilter was calculated using the formula:

Tilter (cfu/mL) = No. of colonies × dilution factor × 10

Twenty isolated clones were selected randomly and amplified by PCR. The mixtures were denatured at 94℃ for 3 min, followed by amplification for 30 cycles: 94℃, 1 min, 52℃, 1 min, 72℃, 3 min. Amplified product (5 µL) was analyzed by 1.0% agarose gel electrophoresis, followed by ethidium bromide staining.

Sequence analysis of identified cDNA clones

The amplified cDNA products of randomly selected positive clones were purified and the nucleotide sequence of cDNA inserts was determined by Takara-corporation. The sequence alignment was completed using the software of BLASTN from the World Wide Web http://www.ncbi.nlm.nih.gov/blast.

Expression profile analysis

Total RNA was isolated from different organs of white flower *S. miltiorrhiza*, including roots, stems, leaves and flowers, to investigate the *SmMYB*90, *SmPAL*1, *SmHMG*R, *Sm4CL*1 and *SmCHS* expression profile. One set of the leaves samples were treated under 100 µM MeJA. Leaves were harvested after 4, 8 and 12 h of treatments respectively using untreated leaves as control. Expression profiles of these genes in different organs and under different treatments were analyzed by RT-PCR using 400 ng RNA as template. 18S rRNA gene was used as internal control.

RESULTS AND DISCUSSION

RNA isolation

Isolation of intact, high-quality RNA from plant tissues is the basis for cDNA library construction. Difficulties extracting high-quality RNA from recalcitrant plant tissues are often due to high levels of phenolics, carbohydrates, or other compounds that bind and/or coprecipitate with RNA in medicinal plants such as *S. miltiorrhiza bge* (Lewinsohn et al., 1994). In the present study, we used modified CTAB method for isolating RNA from root of white flower *S. miltiorrhiza*. The 0.5 g/L spermidine and 0.5% bentonite in CTAB extraction buffer, higher concentration of KAc (pH < 5. 8, 5 mol/L) and high concentration of LiCl (8 M) are efficient for high quality RNA extraction. The yield of RNA was 356.12 µg/g FW. Distinct 28S and 18S ribosomal RNA bands were found in ethidium bromide staining 1.0% denaturing agarose gel and the brightness of 28S is probably as twice as that of 18S RNA (Figure 1). These showed that RNA was relatively free of RNases and has good integrity. It could be explained by that polysaccharides can be removed effectively by low concentration ethanol and higher concentration of KAc co-precipitation in root of *S. miltiorrhiza*, similar to the result of Chun et al. (2008). Bentonit was used in the CTAB extraction buffer because it can adsorb protein and inhibit RNAase (Thoresen et al., 1983; Dunn and Hitchborn., 1965). Results showed that the use of bentonit enhanced the integrity and purity of RNA, and also shorten the time of RNA preparation.

In addition to high integrity, we also found that soluble PVP at 2% (w/w) and RNA precipitated by ethylene glycol monobutyl ether instead of isopropyl alcohol were efficient for removing phenolic compounds (Manning, 1991). RNA isolated from roots of *S. miltiorrhiza* precipitated by ethylene glycol monobutyl ether is high purity. The A_{260}/A_{230} ratio of samples was 2.11 indicating that no contamination by polysaccharides or polyphenol occurred. The A_{260}/A_{280} ratio was 1.91 indicating no protein contamination. Purity and integrity assay indicated that RNA prepared by this CTAB modified method is high-quality and is suitable for making cDNA libraries, isolating genes, or investigating gene expression profiles.

High-quality mRNA is critical for constructing a cDNA library. In our study, the ratio of A_{260}/A_{280} to the mRNA was 1.90, and the concentration was 1.28µg/µL. It show-

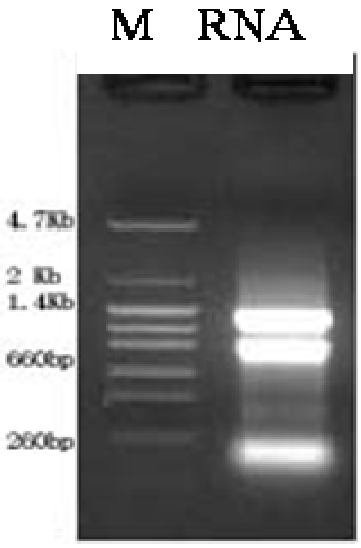

Figure 1. 1.0% agarose gel electrophoresis of total RNA. M: marker.

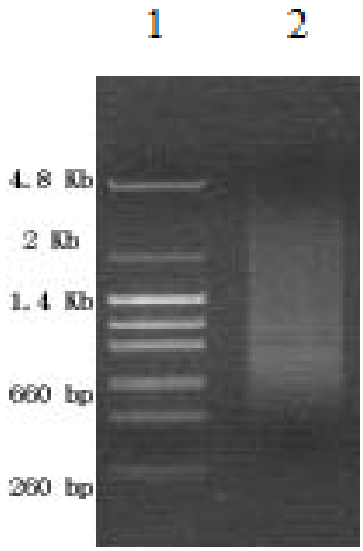

Figure 2. 1.0% agarose gel electrophoresis of fractionated cDNA. 1, DNA marker; 2, fractionated cDNA.

ed that the mRNA obtained from root of white flower *S. miltiorrhiza* did not degrade (Li et al., 2006).

Synthesis and purification of cDNA

SMART technique is a novel and useful method for constructing cDNA libraries. Its important characteristic is that it provides a method for producing high-quality and full-length cDNA libraries that preserve the complete 5′ terminal sequence of mRNA. In this method, as the superiority of LD-PCR in cDNA synthesis, the amount of available RNA starting material needed is very small (i.e., 50 ng of total RNA or 25 ng of mRNA), which is significant to those limited by difficult gaining samples.

Using the SMART cDNA synthesis and long-distance PCR (LD-PCR) amplification strategy (Barnes, 1994), a cDNA library was constructed from mRNA of two-year old root of white flower *S. miltiorrhiza*. The majority of the cDNA produced from LD-PCR was analyzed on a 1.0% agarose gel and a homogeneous smear of PCR product ranging from 300 to 2500 bp in size was observed.

Subsequently, the synthesized cDNA was purified by CHROMA SPIN2400 column to remove short cDNA (less than 400 bp) and adaptor dimmers. Figure 2 showed that the smear of the fractionated cDNA mostly distributed longer than 0.5 kb. The purified cDNAs were used for cDNA library construction.

cDNA library construction

After small-scale reactions the following reaction was determined to be optimal: 1.5 µL cDNA, 1.0 µL pDNR-LIB vector, 0.5 µL ligation buffer, 0.5 µL ATP (50 mM), 0.5 µL T4 DNA ligation and addded water to total volume of 5 µL. After ligation in 16°C for 16 h, the ligation products were transformed into DH10B cell and the cDNA library was constructed. The titer of the constructed cDNA library was approximately 1.8×10^7 cfu/mL and the percentage of recombinants selected from 150 independent clones was 91%. It showed high recombination efficiency. In order to examine the length of the insert fragments, twenty positive cDNA library clones were randomly selected and amplified by PCR using the universal primers. From Figure 3 we could see that the insert fragments of the cDNA library ranged from 600 bp to 2 kb, with an average size of 1000 bp.

Concerning the capacity, the constructed cDNA library must contain at least 1.7×10^5 recombinants (Sambrook and Russell, 2002). In the present study, SMART technique was used to construct cDNA library of white flower *S. miltiorrhiza*, resulting in 1.8×10^7 cfu/ml capacity of the cDNA library, which completely meets the need of cDNA library capacity. In this result, the length of inserted cDNA fragment is about 1 Kb in our result, which also gives high feasibility for cloning full length cDNA.

cDNA sequence analysis

Construction of cDNA expression library is an important molecular biological technique. Through sequencing clones of cDNA library, researchers cannot only identify some known genes but also obtain some novel genes. To understand the characteristics of the cDNA library, 820 clones were randomly selected for sequencing and 756 good EST sequences were obtained. Following BLASTN searches, 70.2% of the inserts were found to be homo-

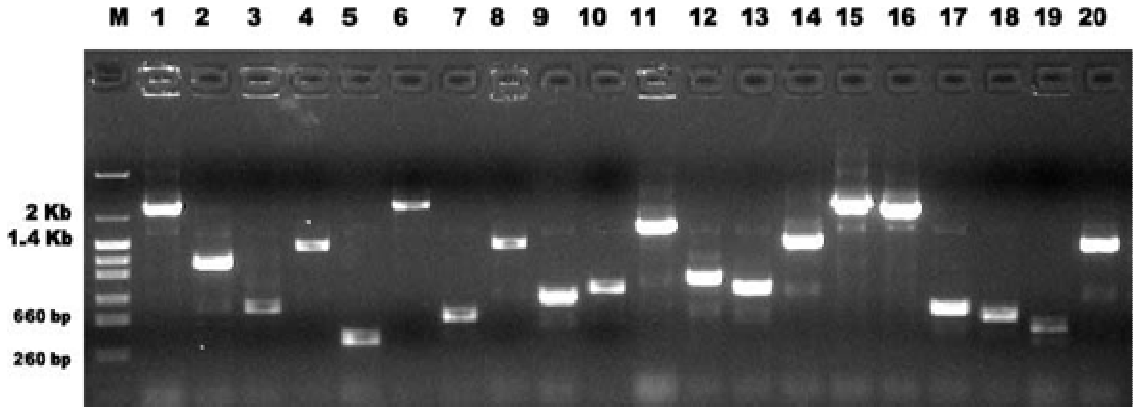

Figure 3. Insert fragment analysis of the recombinant cDNA libraries by PCR amplification. M: DNA marker; 1-20: insert fragments.

logous to sequences in the database. Some ESTs are listed in Table 1. Of these, 67% had a score greater than 150, indicating significant homology to sequences from other organisms (Table 1), including 61% homo-logous to plant sequences. It was noticeable that approxi-mately 30% of genes in the library were not known from a functionally perspective including greater than 8.2% of genes that were likely novel because there was less than 10% sequence identity (non-significant matches) to other species entries in the Genbank and other database (Figure 4). As expected, in addition to significant homologies with plant-specific genes, the strongest matches were also homologous with house keeping genes, e.g. genes encoding ribosomal proteins, translation initiation factors, ubiquitin related protein, energy related protein, and the cellular cytoskeleton (Table 1). Among them, ribosomal related gene is 18.1% (Figure 4).

ESTs related to cell growth and development, such as ADP-ribosylation factor and microtubule associated protein (Table 1), is about 9.4% (Figure 4). ADP-ribosylation factors (ARFs), which serve regulatory roles in vesicular traffic, lipid metabolism, microtubule dyna-mics, development, and likely other cellular processes, are a family of highly conserved proteins within the Ras superfamily of regulatory, monomeric GTP-binding proteins (Boman and Kahn, 1995). In *Arabidopsis*, over-expression of anti-sense ARFA1 reduces cell division, cell expansion, and cellulose production, processes that directly depend on vesicle trafficking for processes such as cell wall construction, which leads the antisense plants severely stunted (Gebbie et al., 2005). Especially, it has been reported that anti-sense suppression of potato ARF produced limited morphological changes (Szopa and Sikorski, 1995), but cAMP levels were reduced (Wilczynski et al., 1997), and sucrose accumu-lation and the decrease in flavonoid and glycoalkaloids level were found to be characteristic features of all transgenic potato plants (Zuk et al., 2003). This suggested that ARFs may be involved in regulation of plant secondarymetabolites biosythesis.

BLASTN searches results showed that 12.8% ESTs are involved in environmental stress response, such as pathology, cold, wound, and heavy metal stress (Figure 4). For example, an EST is similarity to wound-responsive protein, two ESTs for heat shock protein genes, one for a cold acclimation associated sequence and three that may be involved in either oxidative or chemical stress or both (Zn/Cu-superoxide dismutase SOD; selenium-binding protein; glutathione peroxidase-GPX; glutathione S transferase-GST) (Table 1). It is also noteworthy that four EST is associated with pathogen-related protein, such as pathogen-related protein STH-2, legume lectin related protein, PR-10 protein and pathogenesis-related protein (Table 1). Moreover, the frequency in library of these pathogenesis related proteins is higher than other ESTs. Many studies have shown that the synthesis of secondary metabolites in plants is widely believed to be part of the responses of plants to environmental attack. These secondary metabolites are related to plant defense responses, though they are not involved in plant growth and development directly (Xu et al., 2005; Hahlbrock et al., 2003). Therefore, plants may enhance their resistance to stresses by activating secondary metabolite biosynthesis under stresses, such as pathogen, drought and cold, etc (Xu et al., 2008). So, we propose that the roots of *S. miltiorrhiza* should have more secondary-metabolite biosynthesis for defense responses. Thus, gene related to secondarymetabolite biosynthesis in roots should be expressed at higher levels in the roots of *S. miltiorrhiza*.

More importantly, 19.4% ESTs are related to cell metabolism such as sugar metabolism, ammonic acid metabolism and secondary metabolism (Figure 4). Among this category was an EST with similarity to alcohol dehydrogenase, one EST for NAD(P)H-quinone oxidoreductase, one EST for NADP-malic enzyme, and one EST for threonine synthase. Especially, some key enzymes and factors involved in plant secondary metabolites were found from this library (Table 1). Chorismate synthase is an enzyme involved in the biosynthesis of

Table 1. Partial known genes identified from the white flower *Salvia miltiorrhiza* bge. f.alba cDNA library.

BLASTN targeted gene	Frequency in library	Score	Identity (%)
peptidoglycan recognition protein-D	1	267	142/144 (98)
Eukaryotic translation initiation factor 5A	1	301	148/154(96)
60S ribosomal protein L24	5	214	105/112(93)
elongation factor	3	368	179/187(95)
β-tubulin	1	614	329/367(89)
Epl2 protein	2	252	132/137(96)
ubiquinol--cytochrome-c-reductase-related protein	1	221	154/172(89)
Polyubiquitin	2	303	156/157(99)
mitochondrial ATP synthase 6 KD subunit	1	196	138/198(73)
Photosystem I assembly protein Yc4	1	228	109/115(94)
Microtubule associated protein	1	186	93/108(86)
ADP-ribosylation factor	7	218	486/547 (88)
hsp70-I gene for HSP70	1	356	435/456(95)
heat-shock protein 80 gene	1	298	321/365(88)
cold acclimation protein WCOR413	1	365	287/331(87)
wound-responsive protein	1	156	123/176(69)
homologous to glutathione peroxidases	1	415	397/423(94)
mRNA for glutathione S transferase	1	170	267/345(77)
superoxide dismutase (SOD5)	1	315	418/468 (89)
Thioredoxin H	1	231	165/215(76)
SMLII	6	687	683/686 (99)
PR-10 protein	2	219	251/287(87)
legume lectin related protein	4	298	315/343(92)
pathogen-related protein STH-2 gene	2	654	393/410 (95)
pathogenesis-related protein	7	235	107/142(75)
alcohol dehydrogenase	1	431	904/1134 (79)
PAL	1	4289	293/301 (96)
NAD(P)H-quinone oxidoreductase chain 3, chloroplast	1	199	98/105(93)
xyloglucan endo-1,4-beta-D-glucanase	1	409	193/304(63)
Threonine synthase, chloroplast precursor	1	189	151/198(76)
1-aminocyclopropane-1-carboxylate oxidase	2	187	149/175(85)
Chorismate synthase, chloroplast precursor	1	342	169/203(83)
pyruvate decarboxylase	1	360	196/246(79)
NADP-malic enzyme	1	525	265/306(86)

aromatic compounds (Herrmann, 1995). PAL is an important key enzyme in plant secondary metabolism, which catalyzes the first step in phenylpropanoid biosynthetic pathway (Jones, 1984). RT-PCR analysis with RNA from roots, leaves, stems and flowers, revealed that *SmPAL*1 expressed constitutively in all examined tissues but most highly in leaf and roots (Figure 5). CHS catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate. It is the seventh enzyme of the shikimate pathway, which is responsible for the biosynthesis of aromatic metabolites from glucose (Bornemann et al., 1995). RT-PCR analysis of *SmCHS* transcripts showed maximal expression level in flower and a low but detectable level of expression in stems (Figure 5). 1-Aminocyclopropane-1-carboxylate oxidase (ACO) catalyses the last step of ethylene biosynthesis (Ramassamy et al., 1998). HMGR is a key enzyme in the synthesis of IPP in cytosol, which irreversibly catalyzes the formation of mevalonate, one of the known precursors of IPP (Bach, 1995). The transcript level of *SmHMGR* in roots is more than in other tissues (Figure 5). 4CL is an enzyme that functions early in the general phenylpropanoid pathway by producing the monolignol precursor *p*-coumaroyl-CoA. This metabolite is also a precursor for the production of secondary plant metabolites such as stilbenes and flavonoids (Boudet, 2007). RT-PCR results showed that the expression of *Sm4CL*1 could be detected in all tissues, suggesting that *Sm4CL*1 is constitutively expressed but at different levels in different organs (Figure 5). Many studies have shown that

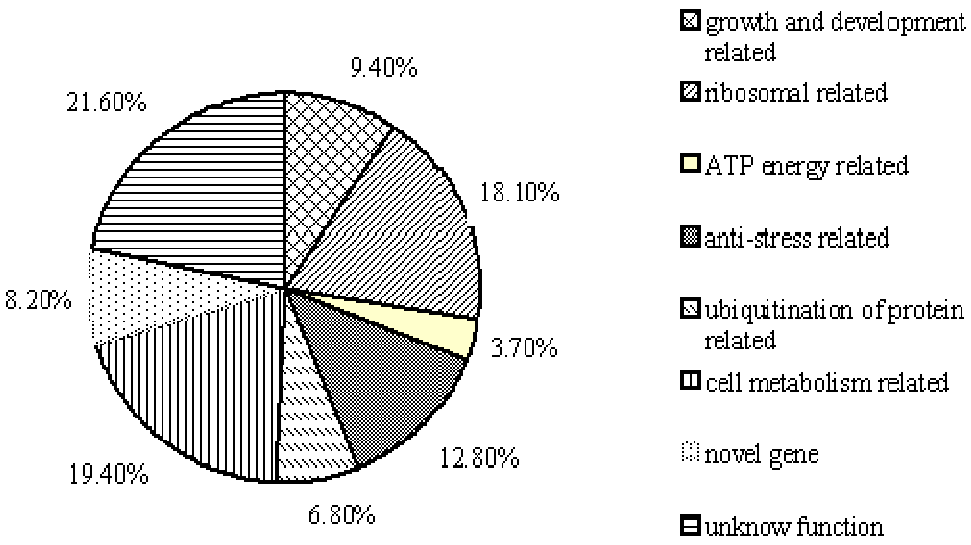

Figure 4. Gene composition in the cDNA library. After cDNAs were cloned into pDNR-LIB vector, 820 positive clones were randomly selected for sequencing. 756 good sequence alignment was completed using the software of BLASTN on the World Wide Web http://www.ncbi.nlm.nih.gov/blast.

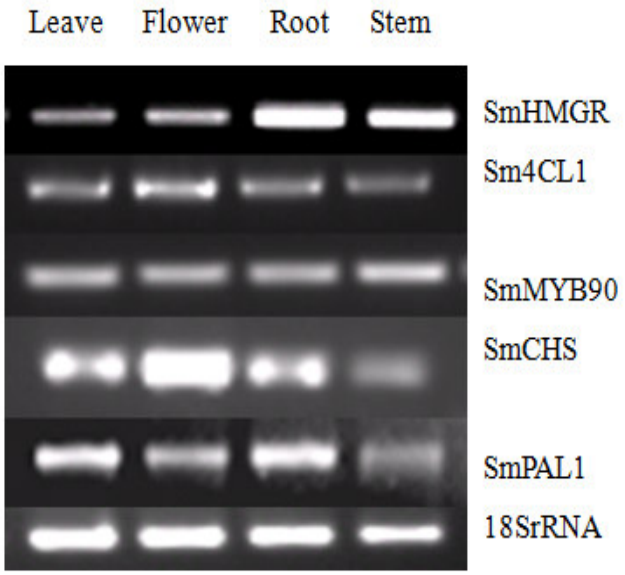

Figure 5. RT-PCR analysis of genes involved in secondary metabolism. RNA was isolated from roots, leaves, stems and flowers of *S. miltiorrhiza*.

R2R3-MYB-related proteins activate the transcription of structural genes that function in different branches of phenylpropanoid metabolism (Martin and Paz-Ares, 1997). The blast result showed that this MYB factor found in this library is similar to PAP2 of *Arabidopsis*, so we named it as *SmMYB*90.The transcript level of *SmMYB*90 in different tissues is no difference, suggesting that this factor is constitutively expressed (Figure 5)

Methyl jasmonate and jasmonic acid, collectively referred to as jasmonates, are important regulators involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening, and senescence (Wasternack and Hause, 2002; Wasternack and Parthier, 1997). In addition, jasmonates activate the defense mechanisms of plants in response to insect-driven wounding, various pathogens, and environmental stresses, such as drought, low temperature, and salinity. Recently, jasmonates have also been implied in the signaling pathway mediating induced defense responses in pathogen- or insect-attacked plants. Exogenously applied jasmonic acid and its methyl ester are capable of inducing defense proteins and secondary defense metabolites in a wide range of plant species (Wasternack and Hause, 2002; Wasternack and Parthier, 1997). As expected, methyl jasmonate could also induce *SmMYB*90, *SmPAL*1, *SmHMGR*, *Sm4CL*1 and *SmCHS* expression of *S. miltiorrhiza* in this study but at different levels for different genes (Figure 6).

In summary, we have successfully isolated high integrity RNA and constructed a better cDNA library of *S. miltiorrhiza* root based on analysis of 756 good EST sequence. Some secondary metabolites related ESTs were obtained from this library. Our cDNA library could provide new insights into regulated mechanism of secondary metabolites in white flower *S. miltiorrhiza* and serve as a foundation for molecular regulation of Danshen metabolite production.

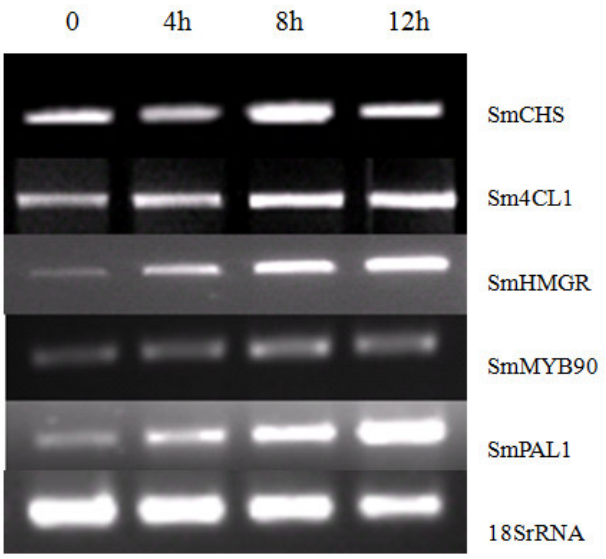

Figure 6. Transcription pattern of genes involved in secondary metabolism induced by methyl jasmonate. RNA was isolated from leaves of *S. miltiorrhiza*.

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