

RESEARCH PAPER

Genome-wide identification and characterization of novel genes involved in terpenoid biosynthesis in *Salvia miltiorrhiza*

Yimian Ma, Lichai Yuan, Bin Wu, Xian'en Li, Shilin Chen and Shanfa Lu*

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, No.151, Malianwa North Road, Haidian District, Beijing 100193, China

* To whom correspondence should be addressed. E-mail: sflu@implad.ac.cn

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Abstract

Terpenoids are the largest class of plant secondary metabolites and have attracted widespread interest. *Salvia miltiorrhiza*, belonging to the largest and most widely distributed genus in the mint family, is a model medicinal plant with great economic and medicinal value. Diterpenoid tanshinones are the major lipophilic bioactive components in *S. miltiorrhiza*. Systematic analysis of genes involved in terpenoid biosynthesis has not been reported to date. Searching the recently available working draft of the *S. miltiorrhiza* genome, 40 terpenoid biosynthesis-related genes were identified, of which 27 are novel. These genes are members of 19 families, which encode all of the enzymes involved in the biosynthesis of the universal isoprene precursor isopentenyl diphosphate and its isomer dimethylallyl diphosphate, and two enzymes associated with the biosynthesis of labdane-related diterpenoids. Through a systematic analysis, it was found that 20 of the 40 genes could be involved in tanshinone biosynthesis. Using a comprehensive approach, the intron/exon structures and expression patterns of all identified genes and their responses to methyl jasmonate treatment were analysed. The conserved domains and phylogenetic relationships among the deduced *S. miltiorrhiza* proteins and their homologues isolated from other plant species were revealed. It was discovered that some of the key enzymes, such as 1-deoxy-D-xylulose 5-phosphate synthase, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, hydroxymethylglutaryl-CoA reductase, and geranylgeranyl diphosphate synthase, are encoded by multiple gene members with different expression patterns and subcellular localizations, and both homomeric and heteromeric geranyl diphosphate synthases exist in *S. miltiorrhiza*. The results suggest the complexity of terpenoid biosynthesis and the existence of metabolic channels for diverse terpenoids in *S. miltiorrhiza* and provide useful information for improving tanshinone production through genetic engineering.

Key words: *Salvia*, *Salvia miltiorrhiza*, tanshinone, terpenoid biosynthesis.

Introduction

Terpenoids, also known as isoprenoids or terpenes, are the largest class of plant secondary metabolites. They play important roles in plant growth, development, general metabolism, and defence against predators, pathogens, and competitors (Gershenson and Dudareva, 2007). Many terpenoids have been used as pharmaceuticals, cosmetics, pesticides, and

Abbreviations: AACT, acetyl-CoA C-acetyltransferase; CDP-ME, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CDP-ME2P, 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; CPS, copalyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; G3P, glyceraldehyde 3-phosphate; GA, gibberellin; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; GPPS, geranyl diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; HMG-CoA, 3-Hydroxy-3-methylglutaryl-CoA; HMGR, hydroxymethylglutaryl-CoA reductase; HMGS, hydroxymethylglutaryl-CoA synthase; IDI, isopentenyl diphosphate isomerase; IDS, isoprenyl diphosphate synthase; IPP, isopentenyl diphosphate; KS, kaurene synthase; KSL, kaurene synthase-like; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; MDC, mevalonate pyrophosphate decarboxylase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MeJA, methyl jasmonate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MK, mevalonate kinase; MVA, mevalonate; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-diphosphate; PMK, 5-phosphomevalonate kinase; TPS, terpene synthase.

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potential biofuels. In the last two decades, the molecular biochemistry and genomics of terpenoid biosynthesis have attracted widespread interest (Bohlmann and Keeling, 2008).

Generally, the biosynthetic pathway of plant terpenoids can be divided into three stages (Fig. 1). The first stage leads to the synthesis of the universal isoprene precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and/or the mevalonate (MVA) pathway. In the second stage, the intermediate diphosphate precursors, including geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), are synthesized under the catalysis of isoprenyl diphosphate synthases (IDSs), including geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS). The last stage involves the formation of diverse terpenoids under the catalysis of terpene synthases/cyclases (TPSs), such as copalyl diphosphate synthase (CPS) and kaurene synthase (KS), and various terpenoid-modifying enzymes. Enzymes involved in terpenoid biosynthesis have different subcellular localizations. All MEP pathway enzymes are located in plastids, whereas the MVA pathway enzymes can be in the cytosol or peroxisomes (Reumann et al., 2007; Sapir-Mir et al., 2008; Simkin et al., 2011). The localizations of IDSs and TPSs are more diversified and often correlated with the subcellular location of terpenoid biosynthesis.

Because of the important role of terpenoids in plant development and the potential value of metabolic engineering of terpenoid biosynthesis pathways, identification and characterization of the genes encoding the enzymes involved

in terpenoid biosynthesis have been carried out in various plant species, such as *Arabidopsis*, conifers, and *Hevea brasiliensis* (Lange and Ghasseman, 2003; Sando et al., 2008a,b; Zulak and Bohlmann, 2010). However, due to the complexity of terpenoid biosynthesis pathways, many enzyme-encoding genes are still not well defined. For example, the terpenoid-modifying enzymes involved in the last stage of terpenoid biosynthesis are largely unknown. In addition, 1-deoxy-D-xylulose 5-phosphate synthases (DXSs) involved in the MEP pathway, hydroxymethylglutaryl-CoA reductases (HMGRs) involved in the MVA pathway, and the IDSs involved in the second stage of terpenoid biosynthesis are encoded by small gene families with at least two members, many of which have not been identified, and the physiological functions of each member are not well known in most plant species. It is particularly true for those species without their whole genome sequence available.

Salvia, including ~900 species, is the largest genus in the economically and medicinally important Labiatae family and is widely distributed throughout the world. Chemical constituents of *Salvia* plants have become a major focus in the related field. Considerable reports about the isolation, identification, structure modification and synthesis, and biology activities of diterpenoids in *Salvia* have been published. The results suggest that *Salvia* produce diverse diterpenoids, such as tanshinone IIA, salvicine, and neotanshinlactone, many of which have significant bioactivities (Honda et al., 1988; Wang et al., 2004; Munro et al., 2005).

Salvia miltiorrhiza Bunge (Danshen in Chinese) is a significant *Salvia* species with great economic and medicinal value. It is the first Chinese medicinal material entering the international market and has been widely used in traditional Chinese medicine (TCM) for treating dysmenorrhoea, amenorrhoea, and cardiovascular diseases (Cheng, 2006). The main lipophilic bioactive components of *S. miltiorrhiza* are diterpenoid tanshinones, including tanshinone I, tanshinone IIA, cryptotanshinone, and so forth. To date, >30 tanshinones and related diterpenoid quinines have been isolated and characterized (Li et al., 2009) and the biosynthesis of tanshinone has been shown to be stimulated by methyl jasmonate (MeJA) treatment in *S. miltiorrhiza* (Gao et al., 2009). These constituents are known to possess a variety of pharmacological effects, such as antibacterial, antioxidant, anti-inflammatory, and antineoplastic activities (Honda et al., 1988). *Salvia miltiorrhiza* has been developed to be a potential model medicinal plant because of its relatively small genome size (~600 Mb), short life cycle, undemanding growth requirements, and significant medicinal value. A total of 13 genes involved in tanshinone biosynthesis have been cloned from *S. miltiorrhiza* (Table 1). Among them, only three, *SmDXS1*, *SmHMGR1*, and *SmGGPPS1*, have been characterized through genetic transformation (Cui et al., 2011). Many genes and gene family members involved in terpenoid biosynthesis are unknown.

Recently, the genome sequencing programme of *S. miltiorrhiza* has been initiated. A working draft of the genome has been obtained (Chen et al., unpublished data). The current assembly has ~20× coverage and consists of 611

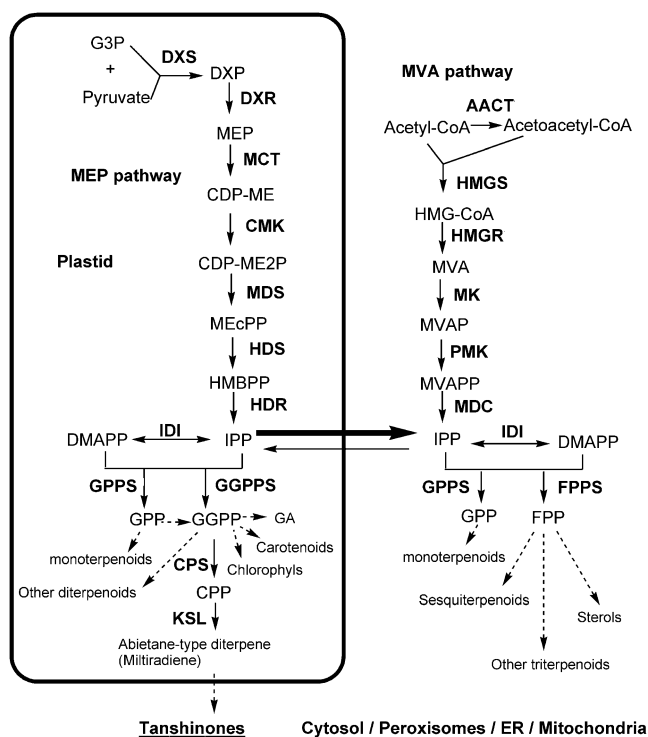


Fig. 1. Proposed pathways of terpenoid biosynthesis in *S. miltiorrhiza*.

Table 1. Terpenoid biosynthesis-related genes in *S. miltiorrhiza*

Name	Accession no.	Len ^a	pl	Mol. wt (kDa)	Loc ^b	TMH ^c	Reference
SmDXS1	EU670744	714	6.37	76.7	C	0	
SmDXS2	FJ643618	725	6.51	78.4	C	0	
SmDXS3	JN831116	713	7.01	76.5	C	0	This study
SmDXS4	JN831117	713	5.93	77.9	C	0	This study
SmDXS5	JN831118	703	6.83	75.5	C	0	This study
SmDXR	FJ476255	474	5.99	51.6	C	0	Wu <i>et al.</i> (2009)
SmMCT	JN831096	304	5.72	33.5	C	0	This study
SmCMK	EF534309	396	6.41	43.4	C	0	Wang <i>et al.</i> (2010)
SmMDS	JN831097	234	8.53	24.6	C	0	This study
SmHDS	JN831098	742	6.02	82.4	C	0	This study
SmHDR1	JN831099	463	5.72	51.9	C	0	This study
SmHDR2	JN831100	462	5.86	52.1	C	0	This study
SmAACT1	EF635969	403	6.33	41.2	–	0	Cui <i>et al.</i> (2011)
SmAACT2	JN831101	403	8.07	41.6	–	0	This study
SmHMGR1	EU680958	565	7.1	60.5	C	2	Liao <i>et al.</i> (2009)
SmHMGR2	FJ747636	550	6.07	58.7	–	2	Dai <i>et al.</i> (2011)
SmHMGR3	JN831102	562	5.74	60.5	–	3	This study
SmHMGR4	JN831103	550	7.51	58.9	–	2	This study
SmHMGS	FJ785326	460	6.04	50.7	–	0	Cui <i>et al.</i> (2011)
SmMK	JN831104	387	5.61	40.8	S	0	This study
SmPMK	JN831095	509	5.3	54.9	–	0	This study
SmMDC	JN831105	422	7.58	46.5	–	0	This study
SmDI1	EF635967	236	5.29	27.2	–	0	Cui <i>et al.</i> (2011)
SmDI2	JN831106	269	5.49	30.6	C	0	This study
SmGPPS	JN831107	424	6.18	46.5	M	0	This study
SmFPPS	EF635968	349	5.63	40.0	–	0	Cui <i>et al.</i> (2011)
SmGGPPS1	FJ643617	364	5.9	39.0	C	0	Unpublished
SmGGPPS2	JN831112	346	6.52	37.4	–	0	This study
SmGGPPS3	JN831113	379	8.35	41.3	C	0	This study
SmGPPS.SSUI	JN831108	314	6.76	34.5	C	0	This study
SmGPPS.SSUII.1	JN831109	290	6.66	31.8	C	0	This study
SmGPPS.SSUII.2	JN831110	331	5.81	36.3	C	0	This study
SmGPPS.LSU	JN831111	344	6.47	37.4	M	0	This study
SmCPS1	EU003997	793	6.04	90.5	C	0	Cui <i>et al.</i> (2011)
SmCPS2	JN831114	757	5.97	86.6	M	0	This study
SmCPS3	JN831115	701*					This study
SmCPS4	JN831120	670*					This study
SmCPS5	JN831121	445*					This study
SmKSL1	EF635966	595	5.7	68.4	C	0	Cui <i>et al.</i> (2011)
SmKSL2	JN831119	762*					This study

^a Len represents the number of amino acid residue. * indicates that the predicted sequence is partial.

^b Loc represents the protein localization predicted by TargetP. 'C' stands for chloroplast, suggesting that the sequence contains a chloroplast transit peptide. 'M' stands for mitochondrial, suggesting the sequence contains a mitochondrial targeting peptide. 'S' stands for secretory pathway, showing that the sequence contains a signal peptide. '–' indicates any other location.

^c TMH represents the number of predicted transmembrane helices.

208 contigs representing ~92% of the entire *S. miltiorrhiza* genome and 96% of the protein-coding genes. It allows a genome-wide identification and characterization of genes involved in terpenoid biosynthesis to be performed.

Materials and methods

Plant materials

Salvia miltiorrhiza Bunge (line 993) with whole genome sequences available was grown in a field nursery. Flowers, leaves, stems, root cortices, and root steles were collected from 2-year-old plants in June when the pharmacologically active components were rapidly

accumulated (Xu *et al.*, 2010). Plant tissues were stored in liquid nitrogen until use.

Plantlets used for MeJA treatment were prepared from stem segments with node and shoot tips of field-grown *S. miltiorrhiza* (line 993). Explants were surface-sterilized in a 0.1% HgCl₂ solution for 5–10 min followed by washing four times in sterile water. The sterilized explants were cultivated on MS agar medium (Murashige and Skoog). Shoots were excised from the explants and rooted on 6,7-V agar medium (Chen *et al.*, 1997) for ~6 weeks under a 16/8 h light/dark photoperiod at 25 °C.

MeJA treatment

Plantlets with regenerated roots were transferred to 6,7-V liquid medium and cultivated for 2 d. MeJA in carrier solution containing

0.1% Tween-20 and 5% ethanol was added to the medium to obtain a final concentration of 200 μ M. Plantlets were treated for 0 h and 24 h and then leaves and roots of similar sizes were collected separately. Plantlets treated with carrier solution were used as controls. Tissues from two individual plants were pooled. All samples were frozen and stored in liquid nitrogen until use.

Sequence retrieval and gene prediction

The current assembly of *S. miltiorrhiza* genome sequences (Chen *et al.*, unpublished data) was searched for homologues of terpenoid biosynthesis-related proteins from various plant species using the BLASTx algorithm (Altschul *et al.*, 1997). An e-value cut-off of 10^{-5} was applied to the homologue recognition. All retrieved sequences were used for gene prediction on the Genscan web server (<http://genes.mit.edu/GENSCAN.html>) (Burge and Karlin, 1998). The predicted gene models were further examined and corrected manually by comparison with related genes identified from other plant species.

Sequence feature analysis

Intron/exon structures were predicted using the Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/chinese.php>) (Guo *et al.*, 2007). The theoretical isoelectric point and molecular weight were predicted using the Compute pI/MW tool on the ExPASy server (Bjellqvist *et al.*, 1994) (http://web.expasy.org/compute_pi/). The localizations of deduced proteins were predicted on the TargetP 1.1 server (<http://www.cbs.dtu.dk/services/TargetP/>) (Emanuelsson *et al.*, 2007). Transmembrane domains were analysed on the TRMHMM server v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) (Krogh *et al.*, 2001). Conserved domains were searched against the Pfam protein families database locally using the Perl script 'pfam_scan.pl' (Finn *et al.*, 2009) (<ftp://ftp.sanger.ac.uk/pub/data/bases/Pfam/Tools/README>). The conserved amino acids were analysed by protein alignment using tools such as ClustalW and then checked manually (Thompson *et al.*, 1994; Hall, 1999).

Phylogenetic analysis

Phylogenetic relationships were analysed using MEGA version 4.0 (Tamura *et al.*, 2007). The Poisson correction parameter and pairwise deletion of gaps were applied. The reliability of branching was assessed by the bootstrap re-sampling method using 1000 bootstrap replications. For each analysis, only nodes supported by bootstrap values >50% are shown.

RNA extraction

Total RNA was extracted from plant tissues using the plant total RNA extraction kit (BioTeke, China) and pre-treated with RNase-Free DNase (Promega, USA) to eliminate genomic DNA contamination. RNA integrity was analysed on a 1% agarose gel. RNA quantity was determined using a NanoDrop 2000C Spectrophotometer (Thermo Scientific, USA).

Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was reverse-transcribed by Superscript III Reverse Transcriptase (Invitrogen, USA). The PCRs were performed according to the instructions of the SYBR premix Ex Taq™ kit (TaKaRa, China) and carried out in triplicate using the CFX96™ real-time PCR detection system (Bio-Rad, USA). Gene-specific primers were designed using primer designing tools such as Primer3 (<http://frodo.wi.mit.edu/primer3/>) (Rozen and Skaletsky, 2000). The primer sequences are listed in Supplementary Table S1 available at *JXB* online. The lengths of amplicons are between 100 bp and 250 bp. *SmUBQ10* was chosen as an endogenous control in this study. The expression of the genes in plantlets treated with MeJA for 24 h was further normalized to their expression in plantlets treated with carrier solution for 24 h. Standard deviations were calculated from

three PCR replicates. The specificity of amplification was assessed by dissociation curve analysis, and the relative abundance of genes was determined using the comparative Ct method as suggested by the CFX-manager software (Bio-Rad, USA).

Results and Discussion

Identification of 40 terpenoid biosynthesis-related genes

Using a systematic computational approach, 40 terpenoid biosynthesis-related genes were identified from the current *S. miltiorrhiza* genome assembly, of which 27 are novel (Table 1). These genes are members of 19 gene families, which encode all enzymes involved in the biosynthesis of the universal isoprene precursor IPP and its isomer DMAPP, and two associated with the biosynthesis of labdane-related diterpenoids (Fig. 1). The exon/intron structures of these genes and the features of deduced proteins were predicted using several web tools (Bjellqvist *et al.*, 1994; Krogh *et al.*, 2001; Emanuelsson *et al.*, 2007; Guo *et al.*, 2007; Finn *et al.*, 2009). These genes have different exon/intron structures. The deduced proteins show different length, isoelectric point (pI), molecular weight, subcellular localization, and transmembrane helix number, and contain conserved domains and motifs (Table 1; Supplementary Fig. S1–S12 and Table S2 at *JXB* online). Phylogenetic analysis shows that these proteins are highly similar to those involved in the biosynthesis of terpenoids in various plants (Supplementary Figs S13–S26). However, tissue-specific expression patterns and different responses to MeJA treatment were found for members in a gene family, suggesting they probably play distinct roles in terpenoid biosynthesis (Supplementary Figs S27, S28). In the following paragraphs, the possible physiological functions of these genes are analysed and discussed in detail based on their sequence features, tissue-specific expression patterns, responses to MeJA treatment, and phylogenetic relationships to homologues in other plant species.

Characterization and expression analysis of genes involved in the MEP pathway

The MEP pathway is mainly present in eubacteria and plants, but it is absent in other eukaryotes, including fungi and animals (Lange *et al.*, 2000). In plants, enzymes involved in this pathway usually operate in plastids to synthesize monoterpenes, diterpenes, carotenoids, and the phytol chain of chlorophyll. Among the 40 identified terpenoid biosynthesis-related genes, 12 encode enzymes involved in the MEP pathway. They include five *DXS* genes, two *HDR* (4-hydroxy-3-methylbut-2-enyl diphosphate reductase) genes, and one each of *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), *MCT* (2-C-methyl-D-erythritol 4-phosphate cytidyltransferase), *CMK* [4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase], *MDS* (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), and *HDS* (4-hydroxy-3-methylbut-2-enyl diphosphate synthase), suggesting that genes encoding all seven MEP pathway enzymes have been identified. Among the 12 genes, seven,

namely *SmDXS3*, *SmDXS4*, *SmDXS5*, *SmMCT*, *SmMDS*, *SmHDS*, and *SmHDR2*, are reported for the first time. Protein subcellular localization prediction indicates that enzymes encoded by these genes are most possibly located in chloroplasts (Table 1). These findings are consistent with the previous reports for *Arabidopsis* MEP pathway enzymes (Hsieh *et al.*, 2008).

DXS is the first enzyme of the MEP pathway. It catalyses the transketolase-type condensation reaction of pyruvate and glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose 5-phosphate (DXP) and plays a critical role in the biosynthesis of terpenoids (Chappell, 1995; Estevez *et al.*, 2001). The present results suggest that DXS is encoded by a small gene family of five members in *S. miltiorrhiza*. The sequences of *SmDXS1* and *SmDXS2* have previously been submitted to GenBank, while *SmDXS3*, *SmDXS4*, and *SmDXS5* are newly identified in this study. All five genes encode proteins with domains and motifs conserved among previously known DXSs. They include the consensus thiamine pyrophosphatase-binding motif and the pyridine-binding DRAG domain, suggesting that SmDXSs have the same type of biochemical activity (Supplementary Figs S2, S3 at *JXB* online).

Previous studies suggested that two different classes of DXS genes existed in plants. Genes in the DXS1 clade are probably involved in primary metabolism, such as the biosynthesis of carotenoids and the phytol chain of chlorophyll, and play housekeeping roles. Genes in the DXS2 clade are probably involved in secondary terpenoid biosynthesis (Walter *et al.*, 2002; Phillips *et al.*, 2007). Recent studies revealed the presence of the third, DXS3, clade. Genes in this clade were proposed to be involved in the biosynthesis of some products essential for plant survival and required at very low levels (Cordoba *et al.*, 2011). Among the five *SmDXS* genes, *SmDXS1* and *SmDXS5* belong to the DXS1 clade, *SmDXS2* and *SmDXS3* are members of the DXS2 clade, while *SmDXS4* is in the more divergent DXS3 clade (Fig. 2A). These results suggest the existence of members of all three DXS clades in *S. miltiorrhiza* and indicate the different roles of each *SmDXS* gene in terpenoid biosynthesis. Consistently, differential expression of *SmDXS* genes was observed. *SmDXS1* is highly expressed in leaves, stems, and flowers. *SmDXS2* is predominantly expressed in leaves, stems, and root cortices. *SmDXS3* is expressed in all the tissues analysed except for flowers. The expression of *SmDXS4* appears to be ubiquitous. *SmDXS5* is mainly expressed in leaves and stems and its expression is low compared with that of the other four *SmDXS* genes (Fig. 2B). These expression patterns of *SmDXS* genes are in agreement with the proposed functions of DXS genes in each clade. In root cortices, which is the main location of major bioactive constituents, such as tanshinones, the level of *SmDXS2* is the highest compared with other *SmDXS* genes, suggesting the importance of *SmDXS2* in tanshinone biosynthesis in *S. miltiorrhiza*. Like *SmDXS2*, *SmDXS3* is one of the DXS2 clade genes that are probably involved in secondary terpenoid biosynthesis. In addition to leaves, stems, and root cortices, where *SmDXS2* is highly expressed,

SmDXS3 is also expressed in root steles (Fig. 2B). These findings, together with the results showing that *SmDXS3* is highly induced by MeJA in the roots of *S. miltiorrhiza* plantlets (Fig. 2C), indicate the involvement of *SmDXS3* in the biosynthesis of defence-related terpenoids in roots. The present results are consistent with those from some other plant species. For instance, both of the *Populus abies* DXS genes (*PaDXS2A* and *PaDXS2B*) belong to the DXS2 clade. However, they are differentially expressed and only one is induced under MeJA treatment (Phillips *et al.*, 2007).

DXR is the second enzyme of the MEP pathway. It is involved in an intramolecular rearrangement and reduction step to form MEP from DXP in the presence of NADPH. Searching the current assembly of the *S. miltiorrhiza* genome, only one DXR gene was found (*SmDXR*). It contains 12 exons and 11 introns (Supplementary Fig. S1 at *JXB* online). *SmDXR* was previously reported to be expressed constitutively and to play a significant role in the MEP pathway, and it was suggested to regulate the production and accumulation of tanshinones in *S. miltiorrhiza* (Wu *et al.*, 2009). In this study, *SmDXR* shows a tissue-specific expression, with the highest level in leaves, followed by stems and flowers. The expression of *SmDXR* in roots is very low, which is consistent with the low level of tanshinones, indicating that DXR is a rate-limiting enzyme of tanshinone biosynthesis (Supplementary Figs S27, S28).

MCT catalyses the conversion of MEP to CDP-ME [2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol] in a CTP-dependent reaction. In plants, the MCT gene was first cloned from *Arabidopsis* and was showed to be a key enzyme in the MEP pathway (Rohdich *et al.*, 2000). However, it has never been isolated from *S. miltiorrhiza* before. Using a computational approach, an *SmMCT* gene was identified from the current genome assembly. The deduced protein contains the conserved IspD motif and shows high identities with other plant MCTs (Supplementary Fig. S2 at *JXB* online). *SmMCT* is mainly expressed in leaves and can also be expressed in stems, roots, and flowers. *SmMCT* is not obviously induced by MeJA, indicating that it is a constitutive gene in *S. miltiorrhiza* (Supplementary Figs S27, S28).

CMK catalyses the phosphorylation reaction of the 2-hydroxy group of CDP-ME and converts it into CDP-ME2P. *SmCMK* has been reported recently (Wang *et al.*, 2010). Analysis of gene expression shows that *SmCMK* is expressed in all tissues analysed, including leaves, stems, roots, and flowers (Supplementary Figs S27, S28 at *JXB* online), which is similar to the expression of *SmMCT*. These results are consistent with the functions of *SmMCT* and *SmCMK* in the biosynthesis of diverse terpenoids.

In the next two steps of the MEP pathway, CDP-ME2P is converted by MDS into a cyclic intermediate MEcPP (2-C-methyl-D-erythritol 2,4-cyclodiphosphate), which is then converted into HMBPP (4-hydroxy-3-methylbut-2-enyl diphosphate) by HDS. The genes encoding MDS and HDS have not been characterized in most plant species. From the *S. miltiorrhiza* genome, an *SmMDS* gene and an *SmHDS* gene were identified and their sequence features were analysed. *SmMDS* contains only three exons, which is the smallest

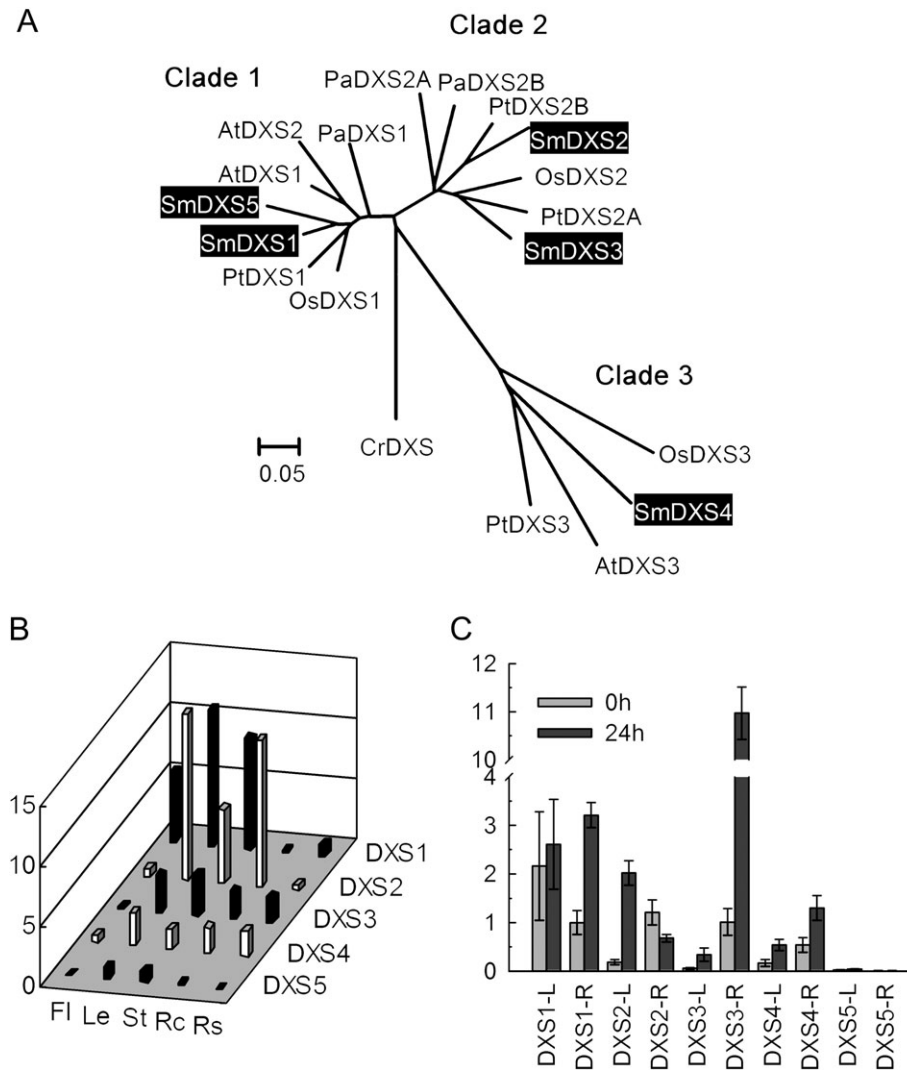


Fig. 2. Expression patterns of *SmDXS* genes and the phylogenetic relationship of their deduced proteins with various other plant species. (A) Phylogenetic relationship of plant DXSs. The rooted Neighbor-Joining tree was constructed using the MEGA program (version 4.0) with default parameters. CrDXS (*Chlamydomonas reinhardtii*, CAA07554) was used as an outgroup. Transit peptides of DXSs were trimmed for the analysis of sequence data. DXSs included are *Arabidopsis thaliana* AtDXS1 (At4g15560), AtDXS2 (At3g21500), AtDXS3 (At5g11380), *Oryza sativa* OsDXS1 (NP_001055524), OsDXS2 (NP_001059086), OsDXS3 (BAA83576), *Populus trichocarpa* PtDXS1 (XP_002312717), PtDXS2A (XP_002303416), PtDXS2B (XP_002331678), PtDXS3 (XP_002308644), *Picea abies* PaDXS1 (ABS50518), PaDXS2A (ABS50519), PaDXS2B (ABS50520), and five *S. miltiorrhiza* *SmDXS*s (highlighted). (B) Fold changes of *SmDXS* genes in flowers (Fl), leaves (Le), stems (St), root cortices (Rc), and root steles (Rs) of *S. miltiorrhiza* plants grown in soil. The expression level of *SmDXS1* in root steles was arbitrarily set to 1. (C) Fold changes of *SmDXS* genes in leaves (L) and roots (R) of *S. miltiorrhiza* plantlets treated with MeJA for 0 h and 24 h. The level of *SmDXS1* in roots of plantlets without treatment was arbitrarily set to 1.

exon number among all 12 MEP pathway genes. In contrast, *SmHDS* has the most complex intron/exon structure, containing 19 exons and 18 introns (Supplementary Fig. S1 at *JXB* online). The expression patterns of *SmMDS* and *SmHDS* are similar to that of other single gene family members of the MEP pathway, showing expression in all tissues analysed and exhibiting slight induction by MeJA.

HMBPP produced under the catalysis of MDS can be further converted into the isoprene precursor IPP by HDR, an enzyme also playing a key role in the supply of plastidial terpenoid precursors (Botella-Pavia et al., 2004). In this study, two *HDR* genes were identified in the *S. miltiorrhiza*

genome. The deduced amino acid sequences of *SmHDR1* and *SmHDR2* show 82.1% identity. The two *SmHDR* genes have similar exon/intron structures, indicating they are probably derived from gene duplication events (Supplementary Fig. S1 at *JXB* online). *SmHDR1* is expressed in all tissues analysed and the expression can be induced by MeJA. In contrast, *SmHDR2* exhibits more tissue-specific expression with very high levels in leaves, flowers, and stems. The expression of *SmHDR2* appears not to be affected in *S. miltiorrhiza* plantlets under MeJA treatment (Fig. 4A, B). These results indicate that *SmHDR1* is probably involved in the biosynthesis of secondary terpenoids, such as tanshinones,

and also plays a significant role in defence responses in *S. miltiorrhiza*, while *SmHDR2* is probably involved in primary metabolism and plays a housekeeping role.

Characterization and expression analysis of genes involved in the MVA pathway

The MVA pathway is an ancestral metabolic route existing in all three domains of life, such as eukaryotes and a few bacteria (Lombard and Moreira, 2011). It mainly operates in the cytoplasm and mitochondria, and predominantly synthesizes sterols, sesquiterpenes, and ubiquinones. A total of six enzymes are involved in this pathway (Fig. 1). From the *S. miltiorrhiza* genome, a total of 10 genes were identified for the six MVA pathway enzymes (Table 1). These include four (*SmA ACT1*, *SmHMGS*, *SmHMGR1*, and *SmHMGR2*) reported previously and six newly identified (*SmA ACT2*, *SmHMGR3*, *SmHMGR4*, *SmMK*, *SmPMK*, and *SmMDC*).

Acetyl-CoA C-acetyltransferase (AACT) catalyses the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. Two AACT genes (*SmA ACT1* and *SmA ACT2*) were identified in the *S. miltiorrhiza* genome. They encode proteins with a conserved thiolase domain (Supplementary Fig. S2 at *JXB* online). *SmA ACT1* and *SmA ACT2* show 76.7% identity at the amino acid level and have the same number of exons and introns (Supplementary Fig. S1). The deduced *SmA ACT1* and *SmA ACT2* proteins are predicted to be cytoplasmic (Table 1), and both of them lack PTS1 peroxisomal targeting sequences in the C-terminus, suggest they could not be localized in peroxisomes (Supplementary Fig. S4). Gene expression analysis shows that *SmA ACT1* and *SmA ACT2* are expressed in all of analysed tissues, with predominant expression in stems. However, the levels of *SmA ACT1* are much higher than those of *SmA ACT2*, suggesting the importance of *SmA ACT1* in *S. miltiorrhiza* (Fig. 4C, D). *SmA ACT1* has previously been reported to be involved in tanshinone biosynthesis (Cui *et al.*, 2011), whereas the exact functions of *SmA ACT2* need to be characterized further.

Hydroxymethylglutaryl-CoA synthase (HMGS) catalyses the condensation reaction of acetyl-CoA and acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). An *SmHMGS* gene was identified from the current assembly of the *S. miltiorrhiza* genome. It contains four exons and three introns (Supplementary Fig. S1 at *JXB* online). *SmHMGS* is expressed in all tissues analysed and is not significantly induced by MeJA (Supplementary Figs S27, S28). *SmHMGS* was indicated to be involved in tanshinone biosynthesis (Cui *et al.*, 2011; Zhang *et al.*, 2011). However, its roles in the biosynthesis of other terpenoids remain to be identified.

HMGR catalyses the conversion of HMG-CoA to MVA, which is the first committed step in the MVA pathway. Although HMGR is encoded by a single gene in higher animals, archaea, and eubacteria, it is usually encoded by multiple genes in plants. This implies that plant HMGR genes have arisen by gene duplication and subsequent sequence divergence (Friesen and Rodwell, 2004). From

the *S. miltiorrhiza* genome, four HMGR genes (*SmHMGR1*, *SmHMGR2*, *SmHMGR3*, and *SmHMGR4*) were identified, two of which have been described in previous studies (Liao *et al.*, 2009; Dai *et al.*, 2011). The SmHMGR proteins show high sequence similarities in the 3'-terminal region encoding the C-terminal catalytic domain, whereas the 5'-terminal region is highly divergent. Consistent with many other plant HMGRs, all of the deduced SmHMGR proteins contain two potential N-linked glycosylation sites (N-X-S/T), two HMG-CoA-binding motifs (EMPVGYVQIP and TTEGCLA), and two NADPH-binding motifs (DAMGMNM and GTCGGG) in the conserved C-terminal catalytic domain. An additional N-linked glycosylation site (NST) associated with the production of elicited defensive compounds is present in *SmHMGR1* and *SmHMGR3* (Choi *et al.*, 1992; Ha *et al.*, 2003) (Supplementary Fig. S5 at *JXB* online). *SmHMGR1* and *SmHMGR4* have the same exon/intron structures and show very high sequence similarity, implying that they are probably duplicated genes within the *S. miltiorrhiza* genome. Similarly, *SmHMGR2* and *SmHMGR3* are probably duplicated genes because they also show a high sequence similarity and both of them contain only one exon (Supplementary Fig. S1). In contrast to DXSs, all of the plant HMGRs are derived from a common ancestor in evolution (Fig. 3A). Significant differential expression was observed for four *SmHMGR* genes. *SmHMGR1* is highly expressed in flowers, followed by stems and root steles, and shows the lowest expression in leaves. *SmHMGR2* is mainly expressed in stems and leaves. Compared with the other three *SmHMGR* genes, *SmHMGR3* is highly expressed in all of the analysed tissues other than flowers. In contrast, *SmHMGR4* is more flower specific and its expression levels in the other four tissues are the lowest among the four *SmHMGR* genes (Fig. 3B). *SmHMGR1*, *SmHMGR2*, and *SmHMGR3* can be induced by MeJA, whereas the level of *SmHMGR4* is more stable in plants treated with MeJA (Fig. 3C). These results are consistent with previous observations for *SmHMGR1* and *SmHMGR2*, indicating that different HMGR isoforms are probably involved in the biosynthesis of different terpenoids (Liao *et al.*, 2009; Dai *et al.*, 2011). In root cortices, which is the main location of tanshinones, the expression of *SmHMGR3* is the highest, followed by *SmHMGR1*. The expression of *SmHMGR2* and *SmHMGR4* in root cortices is very low (Fig. 3B). This suggests the importance of *SmHMGR3* and *SmHMGR1* in tanshinone biosynthesis. In addition, *SmHMGR2* is also likely to be involved in tanshinone biosynthesis, although its expression in the cortex of roots is low. Consistently, it was shown that overexpression of *SmHMGR2* resulted in the enhancement of tanshinone production in cultured hairy roots of *S. miltiorrhiza* (Dai *et al.*, 2011).

Mevalonate kinase (MK), 5-phosphomevalonate kinase (PMK), and mevalonate pyrophosphate decarboxylase (MDC) proteins catalyse the last three steps of the MVA pathway. The plant MK gene was first cloned from *Arabidopsis*, and there is only one in the *Arabidopsis* genome (Riou *et al.*, 1994; Lluch *et al.*, 2000). Consistent with this, an MK gene

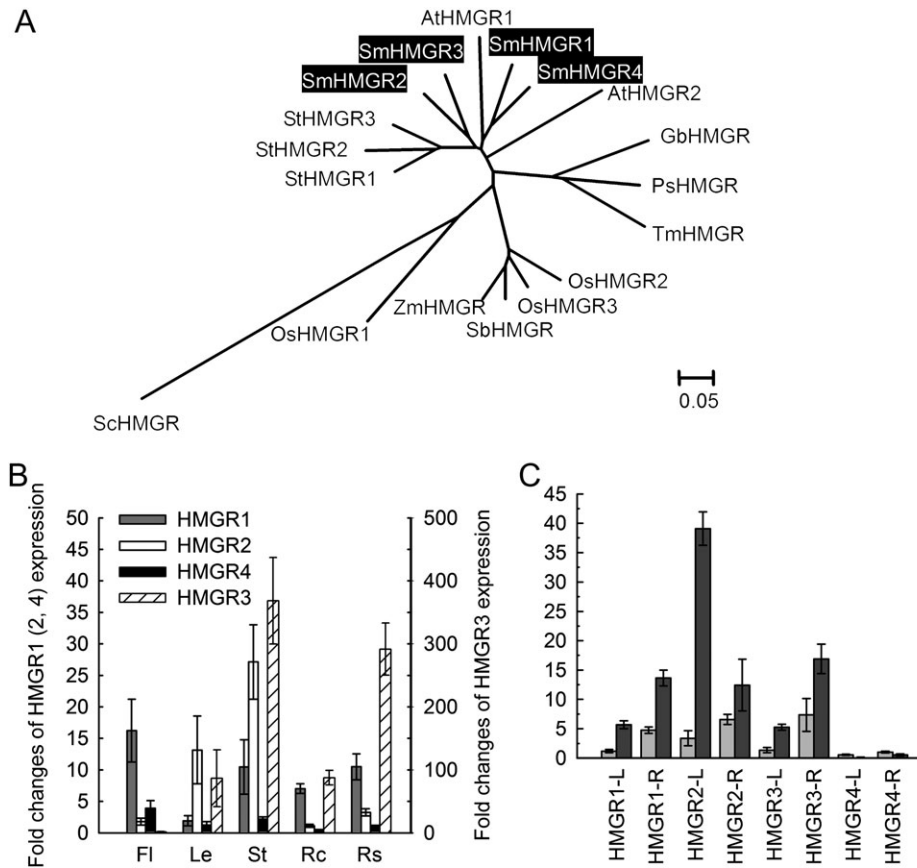


Fig. 3. Expression patterns of *SmHMGR* genes and the phylogenetic relationship of their deduced proteins with various other plant species. (A) Phylogenetic relationship of HMGRs in various plant species. The rooted Neighbor–Joining tree was constructed using the MEGA program (version 4.0) with default parameters. *SchHMGR* (*Saccharomyces cerevisiae*, AAA34676) was used as an outgroup. HMGRs included are *Arabidopsis* AtHMGR1 (CAA33139), AtHMGR2 (AAA67317), *Solanum tuberosum* StHMGR1 (AAA93498), StHMGR2 (AAB52551), StHMGR3 (AAB52552), rice OsHMGR1 (AAA21720), OsHMGR2 (AAD08820), OsHMGR3 (AF110382), *Zea mays* ZmHMGR (O24594), *Sorghum bicolor* SbHMGR (XP_002445887), *Ginkgo biloba* GbHMGR (AAU89123), *Picea sitchensis* PsHMGR (ACN40476), *Taxus×media* TmHMGR (AAQ82685), and four *S. miltiorrhiza* *SmHMGR*s (highlighted). (B) Fold changes of *SmHMGR* genes in flowers (Fl), leaves (Le), stems (St), root cortices (Rc), and root steles (Rs) of *S. miltiorrhiza* plants grown in soil. The expression level of *SmHMGR4* in root steles was arbitrarily set to 1. (C) Fold changes of *SmHMGR*s in leaves (L) and roots (R) of *S. miltiorrhiza* plantlets treated with MeJA for 0 h and 24 h. The level of *SmHMGR4* in roots of plantlets without treatment was arbitrarily set to 1.

was obtained from the current assembly of the *S. miltiorrhiza* genome. *Arabidopsis* MK is preferentially expressed in roots and inflorescences (Lluch et al., 2000), whereas *SmMK* exhibits the highest expression level in stems, followed by root cortices, root steles, leaves, and flowers, and is induced >2-fold in leaves and roots of plantlets under MeJA treatment (Supplementary Figs S27, S28 at *JXB* online), suggesting that MK may have distinct spatial and temporal expression patterns in different plant species. The information about plant PMK and MDC is very limited. Similar to MK, PMK and MDC are also encoded by a single gene and exhibit higher expression level in stems and roots than in leaves and flowers in *S. miltiorrhiza*. The levels of MK and MDC are induced to various degrees by MeJA (Supplementary Figs S27, S28). These results suggest the coordination of *SmMK*, *SmPMK*, and *SmMDC* in the biosynthesis of terpenoids. *SmMK*, *SmPMK*, and *SmMDC* contain the PTS2 peroxisomal targeting signal motif previously found in various MVA

pathway enzymes in other plant species, such as *Catharanthus roseus* (Cr) MK, PMK, and MDC (Supplementary Figs S6–S8) (Simkin et al., 2011). However, the presence of PTS2 in *SmMK*, *SmPMK*, and *SmMDC* does not mean that all of them are peroxisomal enzymes. It has been shown that PTS2 is not necessarily sufficient to target the protein to the peroxisome. For instance, CrPMK and CrMDC are targeted to peroxisomes, whereas CrMK is cytosolic, although they all possess the PTS2 motif (Simkin et al., 2011). Thus, the subcellular localization of *SmMK*, *SmPMK*, and *SmMDC* remains to be determined.

Characterization and expression analysis of *IDI* genes

Isopentenyl diphosphate isomerase (*IDI*) catalyses the reversible conversion of IPP to DMAPP (Ramos-Valdivia et al., 1997). Two *IDI* genes (*SmIDI1* and *SmIDI2*) exist in *S. miltiorrhiza*. *SmIDI1* has been demonstrated to be a

candidate gene involved in tanshinone biosynthesis in hairy roots of *S. miltiorrhiza* (Cui *et al.*, 2011), whereas *SmIDI2* is newly identified. *SmIDI1* and *SmIDI2* have different gene structures and show 65.6% identity at the nucleotide level and 71.5% at the amino acid level. *SmIDI2* possesses a chloroplast localization transit peptide, whereas no such a peptide is predicted for *SmIDI1* (Table 1). Consistently, TargetP prediction suggests that *SmIDI1* is cytosolic, whereas *SmIDI2* is chloroplasmic. In addition, both *SmIDI1* and *SmIDI2* contain the PTS1 peroxisomal targeting signal motif (HKL) (Supplementary Fig. S9 at *JXB* online), suggesting that they are also possibly targeted to peroxisomes. Thus, the localization of *SmIDIs* is complex and need to be analysed further. Gene expression analysis reveals that the transcripts of *SmIDI1* are more abundant than those of *SmIDI2* in all tissues analysed and can be induced to ~2.5 times in leaves and roots of plantlets treated with MeJA for 24 h, suggesting the importance of *SmIDI1* in the biosynthesis of terpenoids (Fig. 4E, F).

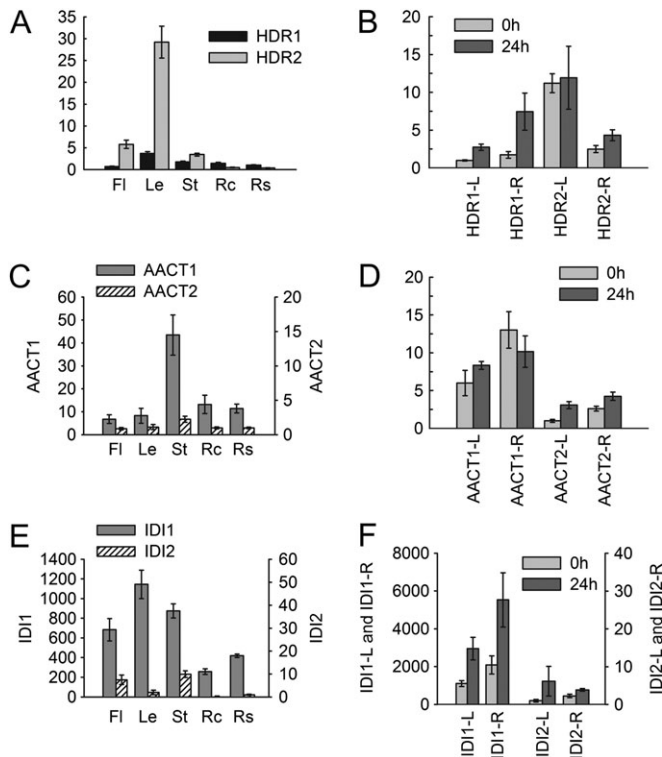


Fig. 4. Expression patterns of *SmHDR*, *SmAACT*, and *SmIDI* genes. (A, C, and E) Fold changes of *SmHDR* genes (A), *SmAACT* genes (C), and *SmIDI* genes (E) in flowers (FI), leaves (Le), stems (St), root cortices (Rc), and root steles (Rs) of *S. miltiorrhiza* plants grown in soil. The expression level of *SmHDR1* (A), *SmAACT2* (B), and *SmIDI2* (C) in root steles was arbitrarily set to 1. (B, D, and F) Fold changes of *SmHDR* genes (B), *SmAACT* genes (D), and *SmIDI* genes (F) in leaves (L) and roots (R) of *S. miltiorrhiza* plantlets treated with MeJA for 0 h and 24 h. The level of *SmHDR1* (B), *SmAACT2* (D), and *SmIDI2* (F) in leaves of plantlets without treatment was arbitrarily set to 1.

Characterization and expression analysis of IDS genes

After IPP and DMAPP formation, the following steps utilize them to form prenyl diphosphates with various chain lengths under the catalysis of IDSs, which are also known as prenyltransferases (PTs). According to the chain length of products, IDSs can be classified into three subfamilies: short-, medium-, and long-chain IDS. Among them, the short-chain IDS subfamily is the most intensively studied. It consists mainly of GGPPS, FPPS, and GPPS. From the current assembly of the *S. miltiorrhiza* genome, nine short-chain IDS genes were identified, three *GGPPS* genes (*SmGGPPS1*, *SmGGPPS2*, and *SmGGPPS3*), one *FPPS* gene (*SmFPPS*), and five *GPPS* genes (*SmGPPS*, *SmGPPS.LSU*, *SmGPPS.SSUI*, *SmGPPS.SSUII.1*, and *SmGPPS.SSUII.2*) (Fig. 5, Table 1). *SmGPPS* and *SmFPPS*, consisting of 12 and 10 exons, respectively, are two *IDS* genes with complex gene structures. The structures of other *SmIDS* genes are much simpler. *SmGGPPS3* probably contains two introns, *SmGGPPS1*, *SmGPPS.SSUII.1*, and *SmGPPS.SSUII.2* contain one intron, while *SmGGPPS2*, *SmGPPS.SSUI*, and *SmGPPS.LSU* are intron free (Supplementary Fig. S1 at *JXB* online). The proteins encoded by *SmGPPS* and *SmGPPS.LSU* are probably localized in mitochondria. *SmGGPPS2* proteins appear to be localized in the cytosol, whereas other deduced IDS proteins are most probably localized in plastids (Table 1). Although all of the deduced IDS proteins contain the conserved polyprenyl_{synthase} domains (Supplementary Fig. S2), the motifs of IDSs could be different. Like other plant GGPPSs and FPPSs, all three *SmGGPPS*s and the *SmFPPS* contain the conserved FARM

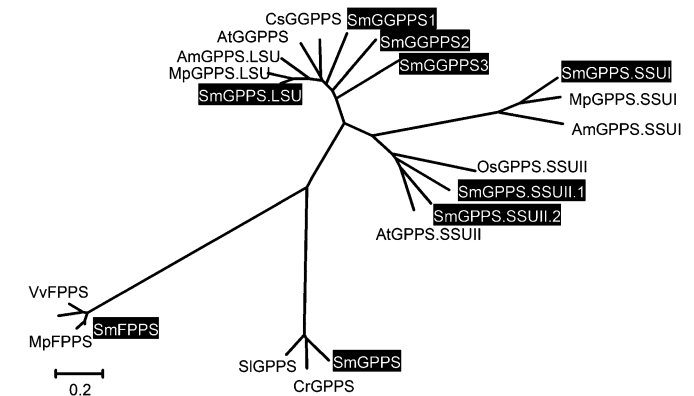


Fig. 5. Phylogenetic analysis of homomeric and heteromeric GPPSs, GGPPSs, and FPPSs in *S. miltiorrhiza* and various other plant species. The unrooted Neighbor-Joining tree was constructed using the MEGA program (version 4.0) with default parameters. Proteins included are *Mentha × piperita* MpGPPS.LSU (AF182828), *Antirrhinum majus* AmGPPS.LSU (AAS82860), *Croton sublyratus* CsGGPPS (BAA86284), *Arabidopsis* AtGGPPS (AAM65107); MpGPPS.SSUI (AF182827), AmGPPS.SSUI (AAS82859), rice OsGPPS.SSUII (EAY87007), AtGPPS.SSUII (At4g38460), *Solanum lycopersicum* SIGPPS (ABB88703), *Catharanthus roseus* CrGPPS (ACC77966), *Hevea brasiliensis* HbFPPS (AAM98379), *Vitis vinifera* VvFPPS (AAX76910), MpFPPS (AF384040), and nine *S. miltiorrhiza* *SmIDS*s (highlighted).

motif (the first aspartate-rich motif, DDX₂₋₄D) and the SARM motif (the second aspartate-rich motif, DDXD), which are important in prenyl-substrate binding (Wang and Ohnuma, 1999). The motifs of GPPSs in plants are more diverse. Interestingly, most of the small subunits of heterodimeric GPPSs discovered in plants contain two conserved CXXXC motifs (where 'X' can be a hydrophobic amino acid, such as alanine, leucine, isoleucine, valine, glycine, or serine) that are crucial for physical interaction between two GPPS subunits (Wang and Dixon, 2009), whereas the large subunit of heterodimeric GPPSs and the subunits of homodimeric GPPSs do not contain these motifs (Supplementary Figs S10–S12; Table S2).

In plants and bacteria, GGPPS catalyses the condensation reactions of IPP and DMAPP to form GGPP, a precursor for the biosynthesis of a structurally diverse group of compounds that includes some specific diterpenoids, carotenoids, chlorophylls, and geranylgeranylated proteins. cDNAs encoding GGPPS have been isolated and characterized from diverse plant species (Okada et al., 2000; Engprasert et al., 2004; Liao et al., 2005). Phylogenetic analysis shows that SmGGPPS1, SmGGPPS2, and SmGGPPS3 proteins are similar to OsGGPPS and AtGGPPS proteins. SmGGPPS1 is expressed in all of the tissues analysed, with high levels in leaves, stems, and root cortices, and the expression level can be induced 2-fold in leaves of plantlets by MeJA (Fig. 6). These results are consistent with the previous report for SmGGPPS1 (Kai et al., 2010). The expression of SmGGPPS1 in *Escherichia coli* has been shown to accelerate the biosynthesis of carotenoids (Kai et al., 2010). Overexpression of SmGGPPS1 in transgenic *S. miltiorrhiza* hairy roots resulted in the enhancement of tanshinone production (Kai et al., 2011). Thus, SmGGPPS1 appears to play a significant role in the biosynthesis of diterpenoids, such as tanshinones that are synthesized mainly in the cortex of roots, and tetraterpenoids, such as carotenoids that are produced in chloroplasts and chromoplasts of plants. SmGGPPS2 and SmGGPPS3 are newly identified genes. SmGGPPS2 is predominantly expressed in the stele of roots, whereas SmGGPPS3 exhibits similar expression levels in all the tissues analysed (Fig. 6). The functions of SmGGPPS2 and SmGGPPS3 remain to be elucidated.

FPPS catalyses the sequential head-to-tail condensation of two molecules of IPP with one molecule of DMAPP to form the sesquiterpenoid precursor, FPP. This enzyme is a homodimer of subunits. The deduced SmFPPS protein shows high identities with known FPPSs isolated from various plants, such as *Mentha × piperita* and *Vitis vinifera* (Fig. 5). Compared with other short-chain SmIDSs, SmFPPS shows the highest expression level in all of the tissues analysed, including flowers, leaves, stems, root cortices, and root steles (Fig. 6A). The expression of SmFPPS can be induced to a higher level by MeJA, particularly in the roots of plantlets (Fig. 6B), indicating the involvement of SmFPPS in defence responses in *S. miltiorrhiza*. SmFPPS was considered to be a candidate gene associated with tanshinone biosynthesis because of the relationship between its expression level and the accumulation of tanshinones (Cui et al., 2011). However, the

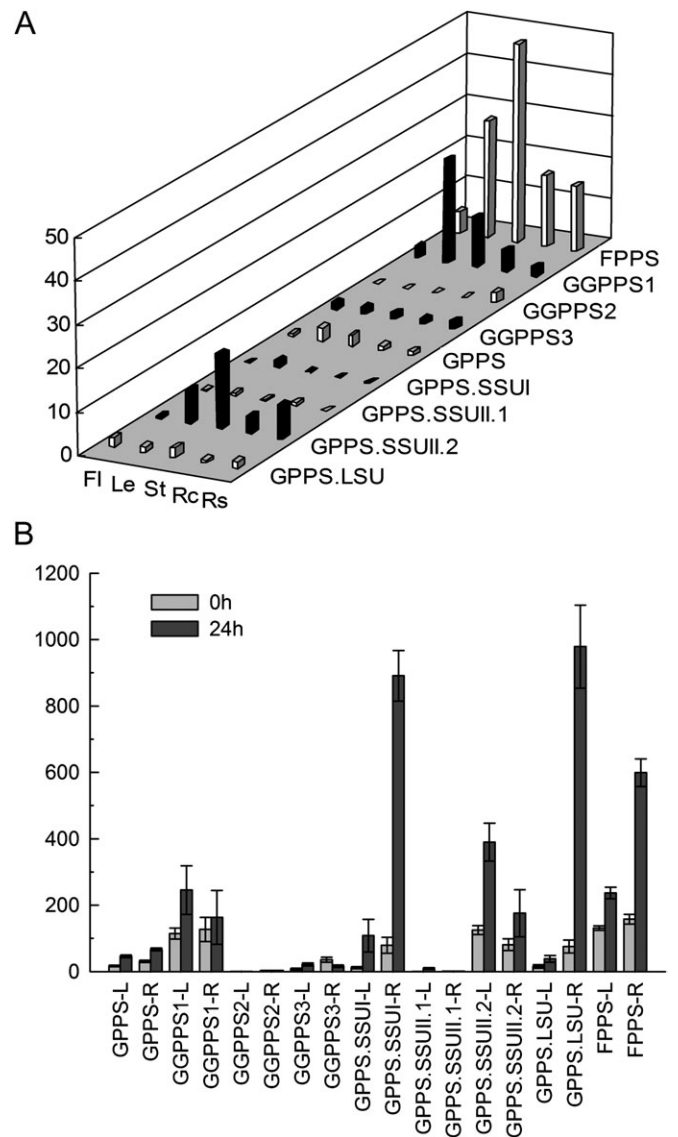


Fig. 6. Expression patterns of SmIDS genes. (A) Tissue-specific expression patterns of SmIDS genes. Fold changes of SmIDS genes in flowers (Fl), leaves (Le), stems (St), root cortices (Rc), and root steles (Rs) of *S. miltiorrhiza* plants grown in soil. The relative abundance of genes is determined using a comparative Ct method, and the expression level of SmGPPS in root steles was arbitrarily set to 1. (B) The expression of SmIDS genes with or without MeJA treatment. Fold changes of SmIDS genes in leaves (L) and roots (R) of *S. miltiorrhiza* plantlets treated with MeJA for 0 h and 24 h. The level of SmGPPS.SSUII.1 in roots of plantlets without treatment was arbitrarily set to 1.

involvement of SmFPPS in tanshinone biosynthesis is very doubtful. No evidence has shown that FPP can serve as a precursor of tanshinones, a group of labdane-related diterpenoids. The increase in SmFPPS expression and the accumulation of tanshinones observed previously are probably two independent events caused by the increase in the IPP level.

GPPS is generally considered to be involved only in monoterpene biosynthesis in plastids, but recent studies show that GPPS is also required for the biosynthesis of

some diterpenoids, such as gibberellins (van Schie *et al.*, 2007). Both homodimeric and heterodimeric GPPSs have been discovered in plants. The homodimeric GPPS exists in both angiosperms and gymnosperms (Bouvier *et al.*, 2000; Burke and Croteau, 2002; van Schie *et al.*, 2007), whereas the heterodimeric GPPS has only been found in some angiosperm plant species, such as snapdragon (*Antirrhinum majus*), *Clarkia breweri*, and hop (*Humulus lupulus*) (Burke *et al.*, 1999; Tholl *et al.*, 2004; Wang and Dixon, 2009). The heterodimeric GPPS is composed of two types of subunits, a large subunit and a small subunit, known as LSU and SSU, respectively (Chang *et al.*, 2010). LSU shows significant homology to homomeric IDSs, such as FPPS and GGPPS, while the homology between SSU and other IDSs is very low. Additionally, two types of SSUs (GPPS.SSUI and GPPS.SSUII) have been described (Wang and Dixon, 2009). In some plants, such as mint, two LSU/SSU heterodimers may form a tetramer (LSU/SSU)₂ to catalyse the production of C₁₀-GPP *in vivo* (Chang *et al.*, 2010). Based on phylogenetic analysis, the five newly discovered *SmGPPS* genes can be classified into *SmGPPS* that encodes the homomeric GPPS subunit and *SmGPPS.LSU*, *SmGPPS.SSUI*, and *SmGPPS.SSUII* encoding heteromeric GPPS subunits (Fig. 5). *SmGPPS.SSUII* is represented by two genes, *SmGPPS.SSUII.1* and *SmGPPS.SSUII.2*, whereas each of the other *SmGPPS* genes is represented by one gene. The discovery of both homomeric and heteromeric GPPSs suggests the complexity of terpenoid biosynthesis in *S. miltiorrhiza*.

SmGPPS.SSUI and *SmGPPS.LSU* show the highest homologies with mint MpGPPS.SSUI and MpGPPS.LSU, respectively, implying the existence of a (LSU/SSU)₂ tetramer in *S. miltiorrhiza* (Fig. 5; Supplementary Fig. S10 at *JXB* online) (Chang *et al.*, 2010). Consistent with the probable role of *SmGPPS.SSUI* in the production of some volatile monoterpenoids, *SmGPPS.SSUI* is predominantly expressed in leaves. The expression of *SmGPPS.LSU* is less tissue specific compared with that of *SmGPPS.SSUI* (Fig. 6A), suggesting that *SmGPPS.LSU* and *SmGPPS.SSUI* are regulated differently in *S. miltiorrhiza*. Both *SmGPPS.LSU* and *SmGPPS.SSUI* can be induced to very high levels in roots of plantlets, indicating the involvement of heteromeric *SmGPPS*s in plant defence responses (Fig. 6B). *SmGPPS.SSUII.2* accumulates to higher levels than *SmGPPS.SSUII.1* in all of the tissues analysed. *SmGPPS.SSUII.1* is predominantly expressed in leaves and root cortices, whereas *SmGPPS.SSUII.2* is highly expressed in stems, followed by leaves and root steles (Fig. 6A), indicating they may be involved in the biosynthesis of different monoterpenoids. The significance of the existence of two types of SSUs (SSUI and SSUII) in a plant species is currently unknown. The aerial parts of many *Salvia* species are covered with trichomes that can produce and accumulate essential oils (volatile oils), a group of monoterpenoid and sesquiterpenoid derivatives with antimicrobial, antioxidant, and antigerminative activities (Bozin *et al.*, 2007; Yousefzadi *et al.*, 2007; De Martino *et al.*, 2010). The biosynthetic mechanism of these essential oils is still unknown. Identification and characterization of sesquiterpenoid and monoterpenoid biosynthesis-related

SmFPPS and *SmGPPS* will definitely help in elucidating the mechanism of essential oil biosynthesis in plants.

Characterization and expression analysis of genes encoding CPS and KSL

CPS and KSL (kaurene synthase-like) are two important terpenoid synthases involved in the biosynthesis of labdane-related diterpenoids. In addition to *SmCPS1* and *SmKSL1* that were reported to be involved in tanshinone biosynthesis (Gao *et al.*, 2009; Cui *et al.*, 2011), a new full-length CPS (*SmCPS2*), three partial CPS genes (*SmCPS3*, *SmCPS4*, and *SmCPS5*), and one partial KSL (*SmKSL2*) were identified through the sequence homology-based search of the current assembly of the *S. miltiorrhiza* genome (Table 1). Full-length sequences of three *SmCPS* genes and a *SmKSL* could not be obtained, probably because the genes involved downstream of the terpenoid biosynthetic pathway are less conserved compared with those involved upstream, as shown in a recent report (Ramsay *et al.*, 2009). It is also probably due to the incomplete *S. miltiorrhiza* genome sequence.

All of the deduced CPS and KSL proteins contain the terpene synthase domains (Supplementary Fig. S2 at *JXB* online). *SmCPS1* and *SmCPS5* exhibit higher expression levels than other *SmCPS* genes in all tissues analysed (Fig. 7). *SmCPS1* is highly expressed in root cortices, followed by stems, root steles, flowers, and leaves. This finding is consistent with previous reports showing the involvement of *SmCPS1* in tanshinone biosynthesis (Gao *et al.*, 2009). *SmCPS5* is predominantly expressed in stems and is also expressed in root cortices, flowers, leaves, and roots. The expression of *SmCPS5* is induced under MeJA treatment in roots of *S. miltiorrhiza* plantlets. These results suggest that *SmCPS5* is also important in terpenoid biosynthesis. Phylogenetic analysis shows that the *SmKSL1* protein is highly similar to tobacco KS, whereas *SmKSL2* is highly similar to *Arabidopsis* and Chinese chestnut KS (Supplementary Fig. S26), implying that the two *SmKSL*s may be functionally distinct. Consistent with this, *SmKSL1* exhibits differential expression patterns, with the highest level in stems, followed by leaves, root cortices, root steles, and flowers, while the levels of *SmKSL2* are similar in all of the tissues analysed (Fig. 7).

Genes probably involved in tanshinone biosynthesis

The biosynthesis of tanshinones is mainly via the MEP pathway, but also depends on cross-talk between the MEP and MVA pathways (Laule *et al.*, 2003; Ge and Wu, 2005). In this study, all of the genes encoding enzymes of the two pathways were systemically characterized in *S. miltiorrhiza*. Among the seven MEP pathway enzymes, five are encoded by single genes (*SmDXR*, *SmMCT*, *SmCMK*, *SmMDS*, and *SmHDS*), whereas the other two, DXS that catalyses the first reaction and HDR that is involved in the last step of the MEP pathway, are encoded by multigene families with five and two members, respectively. Based on gene expression patterns and the results from phylogenetic analysis, it is

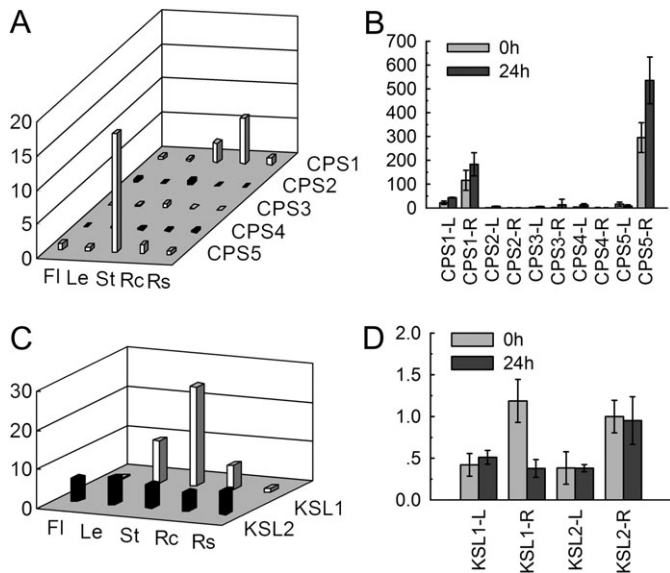


Fig. 7. Expression patterns of *SmCPS* and *SmKSL* genes. (A and C) Fold changes of *SmCPS* genes (A) and *SmKSL* genes (C) in flowers (Fl), leaves (Le), stems (St), root cortices (Rc), and root steles (Rs) of *S. miltiorrhiza* plants grown in soil. The expression level of *SmCPS1* (A) and *SmKSL1* (C) in root steles was arbitrarily set to 1. (B and D) Fold changes of *SmCPS* genes (B) and *SmKSL* genes (D) in leaves (L) and roots (R) of *S. miltiorrhiza* plantlets treated with MeJA for 0 h and 24 h. The level of *SmCPS2* (B) and *SmKSL2* (D) in roots of plantlets without treatment was arbitrarily set to 1.

proposed that *SmDXS2* and *SmHDR1* are probably involved in tanshinone biosynthesis.

Genes encoding the five MVA pathway enzymes in *S. miltiorrhiza* were identified and characterized. HMGS, MK, PMK, and MDC are encoded by single genes, while AACT and HMGR are encoded by small gene families with two and four members, respectively. Compared with *SmaAACT2*, *SmaAACT1* exhibits higher homology with *Arabidopsis* AACT2, which has been suggested to play a role in terpenoid biosynthesis. Thus, *SmaAACT1* has a higher possibility to be associated with tanshinone biosynthesis than *SmaAACT2*. Among the four *SmHMGR* genes, *SmHMGR2* has previously been demonstrated to be involved in tanshinone biosynthesis through genetic transformation (Dai et al., 2011). In this study, it is shown that *SmHMGR3* and *SmHMGR1* are highly expressed in root cortices, the main location of tanshinones. Thus, *SmHMGR1*, *SmHMGR2*, and *SmHMGR3* are probably involved in tanshinone biosynthesis.

Of the two *IDI* genes, *SmIDI1* is probably involved in tanshinone biosynthesis because it shows a much higher expression level than *SmIDI2* and has previously been demonstrated to be associated with tanshinone biosynthesis in hairy roots of *S. miltiorrhiza* (Cui et al., 2011). Among nine short-chain *IDS* genes, *SmGGPPS1* is most probably involved in tanshinone biosynthesis. This gene belongs to the GGPPS subfamily and is highly expressed in the cortex of roots. Overexpression of *SmGGPPS1* resulted in the enhancement of

tanshinone production in transgenic *S. miltiorrhiza* hairy roots (Kai et al., 2011). Moreover, based on expression patterns, *SmCPS1*, *SmCPS5*, and *SmKSL1* appear to be tanshinone biosynthesis-associated terpene synthases.

In summary, a total of 20 genes probably involved in tanshinone biosynthesis have been identified. *SmDXS2*, *SmDXR*, *SmMCT*, *SmCMK*, *SmMDS*, *SmHDS*, and *SmHDR2*, involved in the MEP pathway, seem to play the main role in supplying the isoprene precursor for tanshinone biosynthesis. *SmaAACT1*, *SmHMGS*, *SmHMGR1*, *SmHMGR2*, *SmHMGR3*, *SmMK*, *SmPMK*, and *SmMDC* in the MVA pathway may indirectly affect the supply of IPP precursor. *SmIDI1* and *SmGGPPS1* play significant roles in the second stage of tanshinone biosynthesis, whereas *SmKSL1*, *SmCPS1*, and *SmCPS5* are probably the terpene synthases involved in the third stage of tanshinone biosynthesis. Further characterization of the 40 genes using transgenics may help give a clear picture and add new insights into tanshinone biosynthesis in *S. miltiorrhiza*.

Conclusions

Based on the *S. miltiorrhiza* genome information, 40 terpenoid biosynthesis-related genes were obtained, of which 13 have been reported previously, while the other 27 are novel. These genes can be grouped into 19 families, which include 10 single- and nine multigene families. They encode all of the enzymes involved in the first and second stages of terpenoid biosynthesis and two associated with the third stage. The genomic DNA sequences for these genes were also identified. Using a comprehensive approach, the gene structures and gene expression patterns were analysed. The conserved domains and phylogenetic relationships among the deduced *S. miltiorrhiza* proteins and their homologues isolated from other plant species were analysed. Many unique features of terpenoid biosynthesis-related genes were revealed in *S. miltiorrhiza*. Some of the key enzymes, such as DXS, HDR, HMGR, and GGPPS, are encoded by multiple gene members with different expression patterns and subcellular localizations, suggesting the complexity of terpenoid biosynthesis in *S. miltiorrhiza*. The results support the view that specific groups of terpenoids can be synthesized by specific isoenzymes organized by metabolic channels within the pathway. On the other hand, an isoenzyme may be involved in the biosynthesis of various terpenoids by inserting into different metabolic units (Chappell, 1995; Lluch et al., 2000). Through a systematic analysis, a total of 20 genes were identified that could be involved in the biosynthesis of tanshinones, a group of diterpenoids with significant bioactivities. These results will provide a better understanding of terpenoid biosynthesis in *S. miltiorrhiza* and other plant species and provide the basis for improving tanshinone production through genetic engineering.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Exon and intron structures of 40 terpenoid biosynthesis-related genes.

Figure S2. Conserved domains of enzymes involved in terpenoid biosynthesis in *S. miltiorrhiza*.

Figure S3–26. Sequence alignment and phylogenetic analysis of deduced terpenoid biosynthesis-related proteins from *S. miltiorrhiza* and various other plants.

Figure S27. Expression patterns of 40 terpenoid biosynthesis-related genes in various tissues of *S. miltiorrhiza* plants.

Figure S28. Expression patterns of 40 terpenoid biosynthesis-related genes in *S. miltiorrhiza* plantlets treated with MeJA.

Table S1. Primers used for quantitative real-time RT-PCR.

Table S2. Conserved motifs of IDSs in various plant species.

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