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ORIGINAL ARTICLE

Overexpression of *SmMYB9b* enhances tanshinone concentration in *Salvia miltiorrhiza* hairy roots

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Abstract

Key message **A *Salvia miltiorrhiza* R2R3-MYB gene, *SmMYB9b*, has been cloned and characterized. Overexpression of *SmMYB9b* resulted in a significant improvement of tanshinones, the lipophilic active ingredients in danshen hairy roots.**

Abstract Plant R2R3-MYB transcription factors play important roles in various physiological and biochemical processes. Danshen (*Salvia miltiorrhiza* bunge) is a valuable medicinal herb with tanshinones and salvianolic acids as the principal bioactive ingredients. A number of putative R2R3-MYB transcription factors have been identified in the plant, but their function remains to be studied. Here, we report the cloning of *SmMYB9b,* an S20 R2R3-MYB member and its regulatory properties. *SmMYB9b* contains an open reading frame of 792 bp in length and encodes a 264-amino acid protein. Its transcripts were most abundant in blooming flowers (except for calyces) and increased with flower development. Exogenous abscisic acid strongly activated its transcription. Gibberellins and methyl jasmonate also showed a time-dependent activation effect on its transcription, but to a weaker degree. Overexpression of *SmMYB9b* in danshen hairy roots enhanced tanshinone concentration to 2.16 ± 0.39 mg/g DW, a 2.2-fold improvement over the control. In addition to increased tanshinone concentration, the hairy root growth and lateral hairy root formation were also suppressed. KEGG pathway enrichment analysis with de novo RNAseq data indicated that stress-response-related metabolic pathways, such as the terpenoid and plant hormone signal transduction pathways, were significantly enriched, implying possible implication of SmMYB9b in such processes. Quantitative RT-PCR analysis showed that the transcription of terpenoid biosynthetic genes *SmDXS2, SmDXR, SmGGPPS*, and *SmKSL1* was significantly up-regulated in danshen hairy roots over expressing *SmMYB9b*. These data suggest that overexpression of *SmMYB9b* results in enhanced tanshinone concentration through stimulation of the MEP pathway. The present findings shed new light on elucidating the roles of R2R3-MYB in the biosynthesis of diterpenoids in *S. miltiorrhiza.*

Keywords *Salvia miltiorrhiza* · *SmMYB9b* · R2R3-MYB transcription factor · Tanshinones · MEP pathway

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Introduction

Danshen (*Salvia miltiorrhiza* Bunge) is a widely used medicinal herb with hydrophilic phenolic acids (salvianolic acids, SAs) and lipophilic diterpenoids (tanshinones, TAs) as the principal bioactive components responsible for improving blood circulation and anti-platelet aggregation in clinical applications (Xu 1990; Zhou et al. 2005; Chen et al. 2006; Li and Luo 2008). Salvianolic acids derive

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from phenylpropanoid metabolism (Di et al. 2013; Ma et al. 2013; Petersen 2013; Zhang et al. 2014) and tanshinones are a series of abietane-type diterpenoids synthesized through the terpenoid pathway (Okada 2011). Due to the valuable medicinal properties of these active ingredients, this herb has been the focus of many genomic and transcriptomic studies (Wenping et al. 2011; Gao et al. 2014; Li and Lu 2014; Xu et al. 2016). Recently, a number of R2R3-MYBs have been identified in danshen (Li and Lu 2014).

As one of the largest transcription factor families, R2R3-MYB transcription factors, characterized by a highly conserved MYB domain at N-terminus and an activation or repression domain at the C-terminus, play important roles in various physiological and biochemical processes in plants (Dubos et al. 2010). According to the specific features of the conserved domains, R2R3-MYB proteins are clustered into a number of subgroups based on similar function. For example, R2R3-MYB subgroup four members are involved in the regulation of terpenoid and phenylpropanoid metabolism. Some such examples include, PtMYB14, an R2R3-MYB from *Picea taeda* which is responsible for regulating the biosynthesis of sesquiterpenes and flavonoids in the transgenic conifer tree *Picea glauca* (white spruce) (Bedon et al. 2010), strawberry FaMYB2 has been shown to suppress anthocyanin and flavonol accumulation in transgenic tobacco (Aharoni et al. 2001), AtMYB7 and AtMYB32 demonstrate regulatory roles in flavonoid biosynthesis of *Arabidopsis* (Kim et al. 2015), and PhMYB4/EOBIII acts as a negative regulator in floral volatile benzenoid/phenylpropanoid (FVBP) biosynthesis in petunia (*Petunia hybrida*) (Colquhoun et al. 2011). Usually, the expression of R2R3-MYBs is associated with morphological development in response to internal or external signals such as hormones, light, drought, or other biotic or abiotic stresses (Aharoni et al. 2001; Bedon et al. 2010; Colquhoun et al. 2011; Kim et al. 2015).

By comparison with R2R3-MYBs of other plant species, the members belonging to subgroups S3, S4, S5, S6, S7, S13, and S21 were predicted to be involved in the biosynthesis of salvianolic acids (SAs), while those in subgroups S4, S5, and S20 were assumed to be potential regulators in terpenoid biosynthesis (Li and Lu 2014). Consistent with this deduction, an S4 R2R3-MYB member was shown to be a repressor of rosmarinic acid (RA) biosynthesis (Zhang et al. 2013). Another R2R3-MYB closely related to *Arabidopsis* S5 members, SmPAP1, was identified as an inducer in the accumulation of phenolics and flavonoids in the roots of transgenic *S. miltiorrhiza* plants (Hao et al. 2016). However, to date, most of the other R2R3-MYB members from danshen still remain to be cloned and functionally studied.

Here, we report the isolation of a new S20 R2R3-MYB member, *SmMYB9b,* and its regulatory properties through its overexpression in danshen hairy root cultures. We report markedly increased tanshinone concentrations and an upregulation of a series of stress-responsive metabolic pathways. To the best of our knowledge, this is the first reported R2R3-MYB from *S. miltiorrhiza* with the capability to enhance tanshinone accumulation.

Materials and methods

Plant materials

Danshen (*S. miltiorrhiza* Bunge) were grown in the garden of the Shanghai University of Traditional Chinese Medicine. Wild-type danshen hairy roots were obtained by infecting danshen leaves with *Agrobacterium rhizogenes* LBA 9402. The root cultures were maintained in liquid MSOH medium, a modified Murashige and Skoog medium containing 1 g/l of peptone instead of NH_4NO_3, on an orbital shaker at 120 rpm under dark at 25 °C as described in the previous literature (Hu and Alfermann 1993).

RNA and DNA preparation

Total RNA was extracted from danshen flowers (with corollas without calyces), leaves, roots, stems, calyces, and hairy roots using the Total RNA Isolation Kit (Generay, China) and quantified with a Quawell Q5000 UV–Vis spectrophotometer (USA). cDNA was synthesized using RT^{TM} All-in-One Master Mix (HerogenBio, China) with 1 µg of total RNA in 10 µl of reaction by incubating at 42 °C for 50 min according to the manufacturer's instruction. Genomic DNA was extracted from the leaves of danshen using the Plant DNA Isolation Kit (Generay, China).

cDNA and genomic DNA cloning of SmMYB9b from *S. miltiorrhiza*

Degenerate primers, An-F and An-R, were designed based on the conserved regions of reported plant R2R3-MYB. The core fragment was amplified by PCR using An-F and An-R as primers and leaf cDNA as template under a standard PCR program (initial denaturing at 95 °C for 2 min; 35 cycles of 95 °C 30 s, (Tm-3) °C 30 s, and 72 °C 1 min/kb; followed by a final extension at 72 °C for 15 min). PCR products were cloned into pMD-19T vector (TaKaRa, China) and sequenced. Based on the sequence, gene-specific primers were designed and used for the 5′- and 3′-rapid amplification of cDNA ends (RACE) to get the 5′- and 3′-end fragments (Invitrogen, USA). The full-length

cDNA and genomic DNA of *SmMYB9b* covering the ORF region were amplified using gene-specific primers designed according to the *SmMYB9b* cDNA sequence with cDNA and genome DNA as the templates, respectively. The PCR products were cloned into pMD-19T vector and eight positive clones were randomly picked and sequenced. All primers used in this study were listed in Table S1.

Plant expression vector construction

The entire coding region of *SmMYB9b* was amplified with primers SpeI-F and BstEII-R using Super HF PCR Master Mix (HerogenBio, China). PCR products were purified, digested, and inserted into pCAMBIA-1302, yielding p35S:SmMYB9b (Fig. S1) which was then introduced into *A. rhizogenes* LBA9402 through triparental mating using *Escherichia coli* harboring pRK 2013 as conjugal helper strain according to the literature (Wise et al. 2006). Positive colonies were selected with antibiotic screening followed by plasmid preparation and enzymatic digestion. The pCAMBIA-1302 vector without GFP (named pCAM) was used as a negative control.

Generation of *SmMYB9b* over expressing hairy roots of *S. miltiorrhiza*

SmMYB9b over expressing danshen hairy roots (named SmMYB9b-OX) were induced with *A. rhizogenes* LBA9402 containing p35S:SmMYB9b as described (Hu and Alfermann 1993). Transgenic hairy roots were screened on MSOH solid media with 100 mg/L of hygromycin B and then selected via colony-PCR amplification for the presence of the ORF region of *SmMYB9b* and *rolC* genes. Primers An-SpeI-F and An-BstII-R were designed to cover the gene sequence and partial vector sequence for *SmMYB9b* amplification. Hairy roots transformed with *A. rhizogenes* LBA9402 containing pCAM vector served as a negative control.

Exogenous hormone application experiments

Abscisic acid (ABA), gibberellins (GA), and methyl jasmonate (MeJA) were separately dissolved in 50% DMSO as a 100 mM stock solution. The concentration of individual hormone and the hormone application time were determined based on preliminary experiments (ABA inhibited the hairy root growth) as well as prior literature (Mohr and Cahill 2007; Song and Wang 2009; Xiao et al. 2009; Cui et al. 2012; Yang et al. 2012; Liang et al. 2013; Ma et al. 2013; Cheng et al. 2015; Hao et al. 2016). Briefly, 1 g of fresh wild-type danshen hairy roots were first cultured in 100 ml of liquid MSOH media for 3 weeks. Then, 100 μM of ABA, GA, or MeJA was supplemented and the hairy root cultures were allowed to grow for additional 2–48 h before they were collected. Hairy roots supplemented with 50% DMSO served as a negative control. Three biological replicates were performed for each treatment.

De novo RNAseq and KEGG pathway enrichment analysis

Five independent SmMYB9b-OX hairy root lines and one wild-type hairy root (control) line were used to do the de novo RNAseq and KEGG enrichment analysis by the Majorbio Corporation (Shanghai, China). Briefly, total RNA was isolated from fresh hairy roots, and then, mRNA was enriched, fragmented, and tag-labeled. First strand cDNA was synthesized via reverse transcription and then sequenced using an Illumina Hiseq 2500 (San Diego, CA, USA). Differential expression genes (DEGs) of SmMYB9b-OX hairy roots were analyzed with the edger software (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) using RNAseq data from wild danshen hairy roots as a reference. KEGG pathway enrichment analysis for the DEGs was conducted using KOBAS (http://kobas.cbi.pku.edu.cn/home.do) with the assembled transcriptome as the background. Fisher exact test was used for the calculation with a false discovery rate (FDR) applied to correct the p value. Pathways with p value below 0.05 were considered to be significantly enriched KEGG pathways in DEGs.

Quantitative real-time PCR

Primers of *SmMYB9b* and the reported danshen terpenoid pathway genes, *SmAACT*, *SmHMGS, SmHMGR*, *SmMK*, *SmPMK*, *SmMDC*, *SmIPPI*, *SmFPPS*, *SmGPPS*, *SmDXS*, *SmDXR*, *SmMCT*, *SmCMK*, *SmMDS*, *SmHDS*, *SmHDR*, *SmGGPPS*, *SmCPS*, and *SmKSL*, were designed using the Primer Premier 6.0 software. The *actin* gene was used as internal reference to normalize the data. The amplification efficiency (E) for each gene was evaluated using six sequentially fivefold diluted leaf cDNA samples and those between 95 and 105% were used in the qRT-PCR assay. All the primers were listed in Table S1.

Quantitative real-time PCR (qRT-PCR) was carried out on a StepOnePlus Real-Time PCR system (ABI, USA) using EvaGreen qPCR Master Mix (HerogenBio, Shanghai, China) in 10 μl reaction containing 1.0 μl of cDNA as template under a standard three step program, initial denaturing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, according to the manufacturer's instruction. Three technical replicates were used for each assay. Data were analyzed using the $\Delta\Delta C_t$ method. For *SmMYB9b* overexpression assay, five

independent transgenic hairy root lines were used as biological replicates. For exogenous hormone analysis, three biological replicates were adopted.

Quantification and identification of tanshinones

Tanshinones of danshen hairy roots were isolated and measured as reported (Zhao et al. 2015). Briefly, approximately 50 mg of lyophilized hairy root powder was weighted and soaked in 5 ml of absolute methanol under sonication for 40 min. The mixture was centrifuged at 12,000*g* for 10 min to get the supernatant, which was recovered, condensed in 1 ml of methanol, and filtered through 0.22 μm membrane before analysis (Hu et al. 2005; Chen et al. 2006). Reference standards of cryptotanshinone (CT), tanshinone I (TA-I), and tanshinone IIA (TA-IIA) (Shanghai R&D Center for Standardization of Chinese Medicines, China) were used to determine the concentration of the corresponding compounds. Total tanshinones were the sum of CT, TA-I, and TA-IIA. LC–MS was applied to identify the other diterpenoid compounds.

HPLC–PDA–ESI–MSn conditions

HPLC analysis was carried out with a Beckman Coulter™ ODS column (250 × 4.6 × 5 mm). The program was: 0–25 min, solvent A (acetonitrile) 60%, solvent B (water) 40%; 25–26 min, A 60–80%, B 40–20%; and 26–35 min, A 80%, B 20%. The injection volume was 10 μl at a flow rate of 0.8 ml/min and the detection wavelength was 270 nm.

MS analysis was performed using an LCQ ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with an Xcalibur™ workstation. The positive-ion mode for MS analyses was selected under the following conditions: capillary voltage 19 V, spray voltage 5.0 kV, capillary temperature 300 °C, sheath gas flow rate at 40 (arbitrary units), auxiliary gas flow rate at 20 (arbitrary units), and tube lens offset 40 V. The full scan mass spectra were recorded in the range of *m*/*z* 150–800. The isolation width of precursor ions was 1.0 Th. The HPLC/MS data were acquired and processed using the Finnigan Xcalibur 1.3 software provided by the manufacturer.

Bioinformatics analysis

BLASTX was used to identify partial and complete sequences of *SmMYB9b* by comparing the ORF region translated from the nucleotide sequences against protein databases (http://www.ncbi.nlm.nih.gov). Alignment of the deduced amino acid sequence of *SmMYB9b* with the selected MYB TFs was performed with MegAlign (DNAStar Inc., Madison, WI, USA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 with neighbor-joining (N-J) method (Tamura et al. 2013). Statistical branch support was assessed by bootstrap analysis of 1000 replicate data sets in MEGA.

Correlation analysis was performed to explore the relationship between the genes and the compounds applying the SPSS19.0 software (IBM, Armonk, NY, USA) using bivariate analysis.

Results

Cloning, characteristics, and phylogeny assay of *SmMYB9b*

Approximately 170 bp of the core region of a cDNA fragment homologous to MYB transcription factor was initially amplified with degenerate primers An-F and An-R (Table S1). The full-length cDNA (GenBank accession number: JX113685) of this segment was acquired through 5′/3′-RACE based on the sequence of the core region. It was 1207 bp in length encoding a 264-amino acid (AA) protein with a calculated molecular mass of 30.19 kDa and an isoelectric point of 6.17. The 1823 bp of genomic DNA (GenBank accession number: JX113686) contained a 369 bp noncoding region (promoter and 5′-UTR), a 266 bp 3′-UTR, and two introns separating the ORF into three exons (Fig. S2A).

The cloned sequence had 98% identity with *SmMYB9* (GenBank accession number: KF059363) at both nucleotide and amino acid levels thus was named *SmMYB9b* (Fig. S2B). Further analysis of the deduced amino acid sequence showed that except for three extra amino acid residues, Ser^{11}, Arg^{12}, and Asn^{13}, at the N-terminus of SmMYB9b, there was no other difference between SmMYB9b and SmMYB9 among the 264-AA sequence (Fig. S2C). Considering that no SmMYB9 clones had been obtained in this study, it was possible that SmMYB9b was the real SmMYB9 in danshen plants cultivated in our university. In the phylogenic assay, SmMYB9b was placed into Subgroup 20 (S20) of R2R3-MYB family with six SmMYBs and six *Arabidopsis* S20 members (Fig. 1a). Alignment of these S20 members showed that SmMYB9b had one R2 and one R3 domain, and a WxPRL motif (Fig. 1b) which was highly conserved in *Arabidopsis* R2R3-MYB S20 members (Stracke et al. 2001; Mandaokar and Browse 2009; Hantarachot et al. 2012; Li and Lu 2014), indicating that SmMYB9b possibly had similar functions to previously elucidated S20 members such as AtMYB2, AtMYB62, and AtMYB108 in *Arabidopsis* development and stress responses (Abe et al. 2003;

Mandaokar and Browse 2009; Devaiah et al. 2009; Guo and Gan 2011).

Expression profiles of *SmMYB9b* in danshen plant and hairy roots

Quantitative RT-PCR analysis showed that the transcripts of *SmMYB9b* was most abundant in blooming flowers (except for calyces), followed by flower buds, stems, roots, calyces, and leaves (Fig. 2a). Its expression level in flowers was 1.6-, 12.9-, 16.2-, 17.8-, and 143.9-fold higher than that in flower buds, stems, roots, calyces, and leaves, respectively. This result suggests that SmMYB9b possibly functions in regulation of flowering. The higher level in blooming flowers than in flower buds also indicates that it is more active during late stage of flower development. In addition, the expression level of *SmMYB9b* in danshen hairy roots was about 4.4-fold as high as that in the normal roots, implying that the transcription of SmMYB9b was linked with expression of the *rolA, rolB,* or *rolC* genes that were introduced by *A. rhizogenes*, and which are known to code for modulators of signal transduction (Bulgakov 2008).

Generation of SmMYB9b-OX hairy roots

SmMYB9b over expressing hairy roots (SmMYB9b-OX) was generated via *Agrobacterium*-mediated transformation. Through hygromycin resistance screening and PCR selection, 28 transgenic hairy root lines were obtained (Fig. S3). Relative expression level of *SmMYB9b* in all the transgenic hairy root lines was analyzed with qRT-PCR and displayed in a descending order (Fig. S4). As shown, M1, M11, M5, M4, and M13 were the top five high *SmMYB9b* transcription level hairy root lines which were then chosen as independent biological replicates for the next studies.

Morphology and growth of SmMYB9b-OX hairy roots

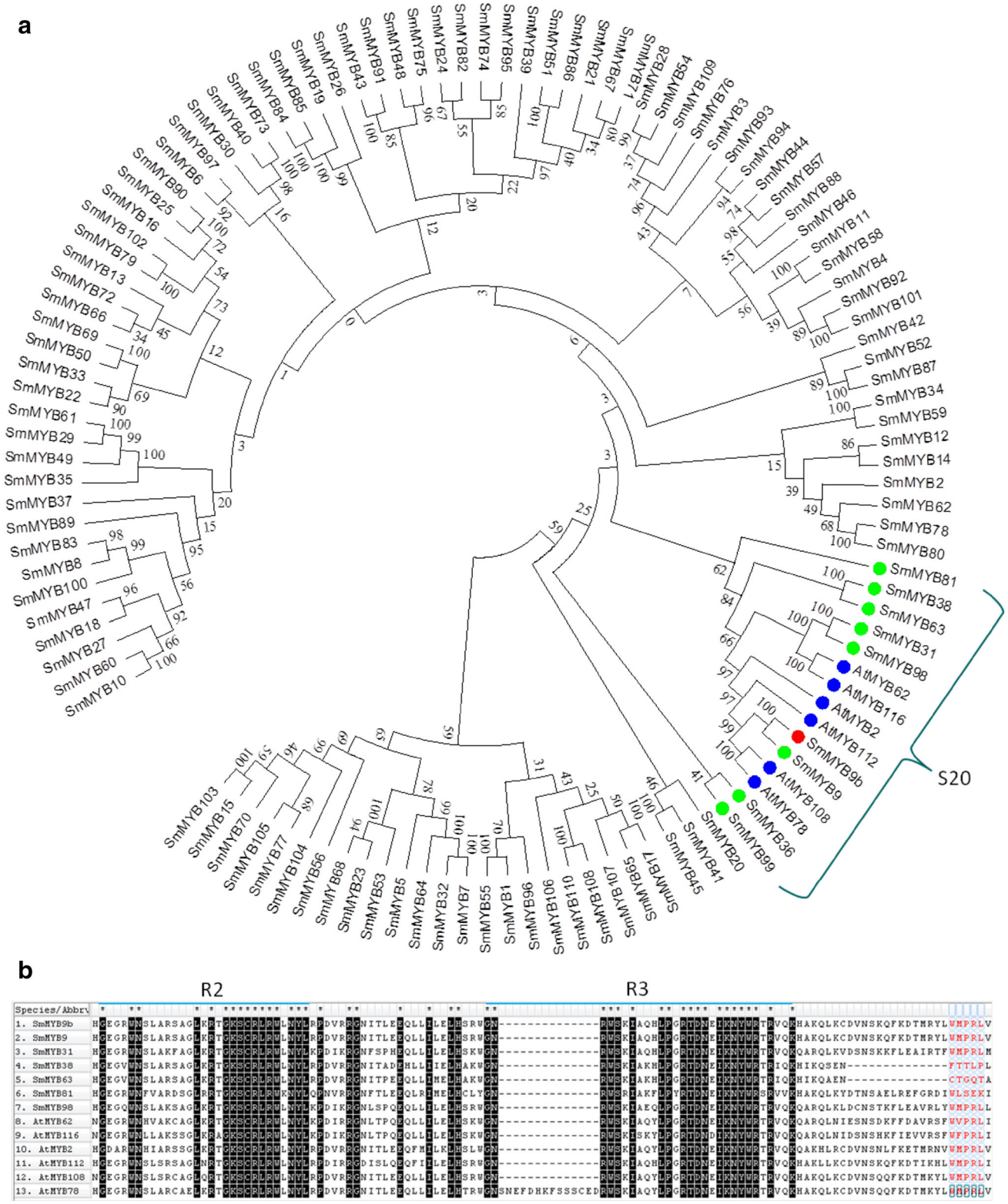
SmMYB9b-OX hairy roots grew thinner with fewer lateral roots than the control when being cultured in liquid MSOH media (Fig. 3a). Particularly, SmMYB9b-OX hairy roots were prone to form callus and further differentiated to buds and shoots when cultured longer than 1 month (Fig. 3b, c). The average biomass of M1, M4, M5, M11, and M13 was 0.34 ± 0.04 g of dry weight (DW), only 34% of the control which was 0.98 ± 0.15 g DW after cultured for 21 days using 1 g of fresh weight (FW) of hairy roots as initial inoculation material (Fig. 3d; Table S2). Pearson correlation analysis using *SmMYB9b* transcripts and the biomass of individual hairy root lines showed that they were negatively correlated with each other ($p = 0.05$, two-tailed) (Table 2). These findings demonstrated that overexpression of *SmMYB9b* suppressed the growth and changed the architecture of danshen hairy roots.

Diterpenoids in SmMYB9b-OX hairy roots

Since S20 R2R3-MYB members had been associated with terpenoid biosynthesis, and tanshinones are the predominant terpenoid derivatives in danshen roots, we analyzed these compounds in SmMYB9b-OX hairy roots, using HPLC–MS. Similar to our earlier study (Zhao et al. 2015), eight major diterpenoids were detected, including 15,16-dihydrotanshinone I (Compound 2), 1,2-didehydromiltirone (Compound 3), methyltanshinonate (Compound 4), cryptotanshinone (CT, Compound 5), tanshinone I (TA-I, Compound 6), 1,2-dihydrotanshinone I (Compound 7), and tanshinone IIA (TA-IIA, Compound 8) (Table S3) with LC–MS, by comparing the fragmentation patterns with those of reference standards, or those of compounds reported in the literature (Hu et al. 2005). As indicated in the typical HPLC profile (Fig. 4), all these compounds were evidently enhanced in SmMYB9b-OX hairy roots, especially 1,2-didehydromiltirone, CT, TA-I, 1,2-dihydrotanshinone I, and TA-IIA compared with the control. The concentration of CT, TA-I, and TA-IIA was 0.29 ± 0.05, 1.45 ± 0.28, and 0.42 ± 0.08 mg/g DW, respectively, leading to 2.16 ± 0.39 mg/g DW of total tanshinones (TTA) in SmMYB9b-OX hairy roots, 2.2-fold of the control (Table 1). Among the five SmMYB9b-OX hairy root lines, M1 yielded the maximum amount of tanshinones at 2.83 mg/g DW of TTA, 2.8-fold of the control (Table S2). Pearson correlation analysis with *SmMYB9b* transcripts and tanshinone concentration in individual SmMYB9b-OX hairy roots showed that CT, TA-I, TA-IIA, and TTA all demonstrated a significant positive correlation with *SmMYB9b* transcripts ($p = 0.05$) (Table 2). These results substantially proved that overexpression of *SmMYB9b* significantly activated tanshinone biosynthesis in danshen hairy roots.

Effect of ABA, GA, and MeJA on *SmMYB9b* transcription

Abscisic acid (ABA), gibberellic acid (GA), and methyl jasmonate (MeJA) were known to induce R2R3-MYBs in other plant species (Urao et al. 1993; Abe et al. 2003; Seo et al. 2009; He et al. 2012; Hao et al. 2016), and overexpression of *SmMYB9b* in danshen hairy roots caused alteration in morphology and tanshinone levels. The effects of ABA, GA, and MeJA on *SmMYB9b* transcription were investigated in wild-type danshen hairy roots. As shown in Fig. 2b, ABA significantly stimulated *SmMYB9b*


a
SmMYB103
SmMYB15
SmMYB70
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SmMYB77
SmMYB104
SmMYB56
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SmMYB23
SmMYB53
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SmMYB64
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AtMYB112
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SmMYB100
SmMYB47
SmMYB18
SmMYB27
SmMYB60
SmMYB10
b
R2
R3
Species/Abbr
1. SmMYB9b
2. SmMYB9
3. SmMYB31
4. SmMYB38
5. SmMYB63
6. SmMYB81
7. SmMYB98
8. AtMYB62
9. AtMYB116
10. AtMYB2
11. AtMYB112
12. AtMYB108
13. AtMYB78

◀**Fig. 1** Phylogeny assay of SmMYB9b with other R2R3-MYB members. **a** Phylogeny assay was conducted using MEGA version 6 with neighbor-joining (N-J) method (Tamura et al. 2013). Statistical branch support was assessed by bootstrap analysis of 1000 replicate data sets in MEGA. SmMYB9b were marked in *red dot*. The six SmMYBs and six AtMYBs S20 members were marked with *green* and *blue green dots*, respectively. The amino acid sequences of *Arabidopsis* and *S. miltiorrhiza* R2R3-MYB members were downloaded from GenBank and grouped according to the literature (Li and Lu 2014). **b** Alignment of SmMYB9b with other S20 members. The completely conserved amino acid residues were highlighted in *black* and the "WxPRL" motif was indicated in *red letters* (color figure online)

transcription. After the addition of ABA, the relative expression level of *SmMYB9b* increased to 27.4-fold of the control after 48 h. GA and MeJA exhibited a much weaker effect on *SmMYB9b* transcription. After treatment with GA, *SmMYB9b* initially increased to 3.6-fold of the control after 2 h, but decreased again and remained at base level (Fig. 2b). After treatment by MeJA, *SmMYB9b* transcripts steadily increased for the first 12 h, reaching the highest level, 3.4-fold of the control, and then declined back to base level. These results indicated that *SmMYB9b* was more sensitive to ABA than to GA and MeJA.

KEGG pathway enrichment analysis and expression profile of enriched pathway genes

De novo RNAseq was carried out to explore the differential expression genes (DEGs) in SmMYB9b-OX hairy roots using wild-type danshen hairy roots as a reference control. A total of 56,041 unigenes were assembled and 1074 of them were determined as significant DEGs in SmMYB9b-OX hairy roots. Pathway enrichment analysis of DGEs was performed based on KEGG database to characterize the functional consequence of alteration in gene expression related to over expressing *SmMYB9b*. Among the 1074 DEGs, a total of 374 of them were annotated as differential expression pathway genes and assigned into 137 KEGG pathways. There were 28 significantly enriched pathways ($p < 0.05$) 21 of which belonged to metabolism (Fig. 5), predominantly showing enrichment in terpenoid- and phenylpropanoid-related pathways, responsible for the biosynthesis of monoterpenoids, sesquiterpenoids and triterpenoids, diterpenoids, carotenoids, and phenylpropanoids, followed by lipid metabolism, namely, cutin, suberine and wax biosynthesis, fatty acid elongation, glycerolipid metabolism, and fatty acid biosynthesis. Plant hormone signal transduction pathways were also significantly enriched.

Due to the specific phenotype of SmMYB9b-OX hairy roots, we were especially interested in the biosynthesis of diterpenoids, carotenoids, and phenylpropanoids, and explored the differential expression genes in these pathways. We found that the transcripts of some annotated genes were markedly up-regulated, including the genes coding for farnesyl diphosphate synthase (FPS) and geranylgeranyl diphosphate synthase (GGPPS) for terpenoid backbone biosynthesis, ent-copalyl diphosphate synthase (CPS), ent-kaurenoic acid hydroxylase, gibberellin 20-oxidase, and gibberellin 3-beta-dioxygenase for GA biosynthesis, (+)-abscisic acid 8′-hydroxylase for ABA metabolism, cinnamate 4-hydroxylase (cinnamate 4-monooxygenase, C4H) for the general phenylpropanoid metabolism, beta-glucosidase for coumarin biosynthesis, cinnamyl-alcohol dehydrogenase (CAD) and peroxidase for lignin biosynthesis, tyrosine aminotransferase (TAT), tyrosine decarboxylase (TDC), and polyphenyl oxidase (PPO) for tyrosine metabolism and flavonoid 3′-monooxygenase for flavonoids biosynthesis.

A couple of annotated plant hormone signal transduction pathway genes were also significantly increased, including genes encoding auxin-responsive protein IAA, auxin response factor, auxin-responsive GH3 gene family, SAUR family protein for auxin signaling, abscisic acid receptor

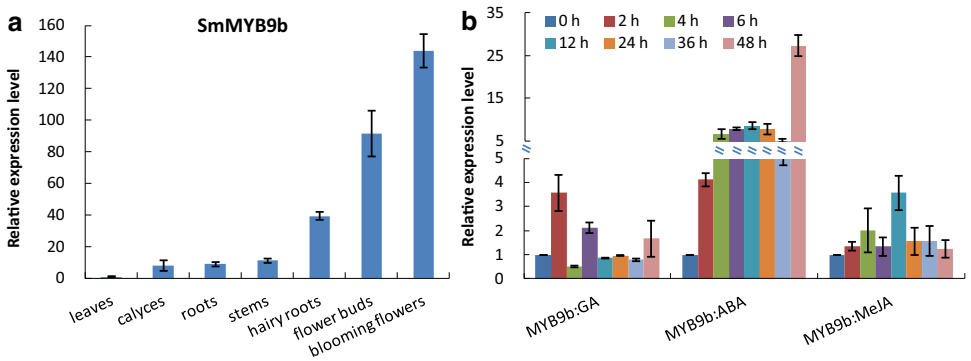

Fig. 2 Expression profiles of *SmMYB9b*. **a** *SmMYB9b* transcripts in different tissues and organs of danshen plants. Danshen leaves were used as the reference sample. All data are the means of three independent replicates with *error bars* indicating the SD. **b** Expression pattern of *SmMYB9b* responding to exogenous application of ABA, GA, and MeJA. The *columns* in *different colors* indicate the hormone treatment time (color figure online)

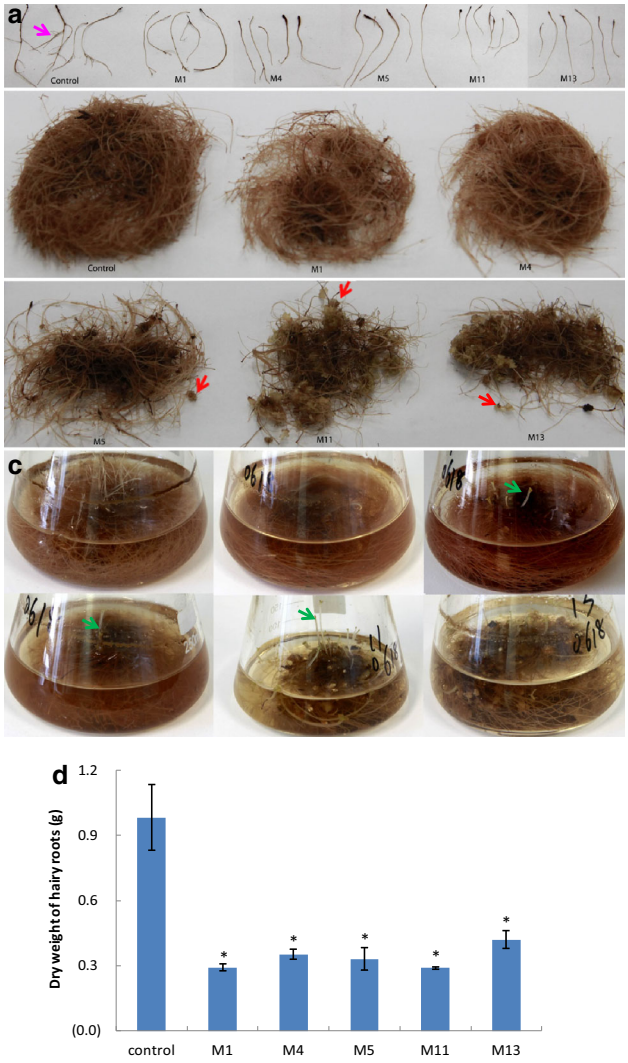

Fig. 3 Typical morphology of SmMYB9b-OX hairy roots. **a**, **b** Hairy roots after being cultured in liquid MSOH media for 21 days. *Pink arrow* indicates the lateral roots of hairy roots. *Red arrows* indicate callus formed during the culture period. **c** Hairy roots cultured in liquid MSOH media for 40 days. *Green arrow* indicates buds and shoots generated from the hairy root cultures. **d** Biomass of SmMYB9b-OX hairy roots cultured in liquid MSOH media for 21 days. The data represent mean ± SD of three replicates for each hairy root line. Student's *t* test was applied to calculate the significant difference between control and the transgenic hairy roots. One *asterisk* above the *columns* indicate the difference between the sample and control is significant ($p < 0.05$) (color figure online)

PYR/PYL family (PYL) for ABA signaling, BR-signaling kinase for brassinosteroid, jasmonic acid-amino synthetase for jasmonic acid, and pathogenesis-related protein 1 for salicylic acid, indicating that plant hormone signaling was involved in overexpression of SmMYB9b-caused hairy root phenotype alterations. These results suggested that a variety of physiological activities were changed at the transcription level in SmMYB9b-OX hairy roots compared with the control.

Expression analysis of terpenoid biosynthetic genes in SmMYB9b-OX hairy roots

To further investigate the expression profile of the terpenoid pathway, we performed qRT-PCR with 24 known genes associated with MEP pathway and MVA pathway using the same samples for tanshinone quantification. As shown in Fig. 6, the transcripts of MEP pathway genes, *SmDXS2, SmDXR, SmGGPPS,* and *SmKSL1,* were significantly increased. For the MVA pathway genes, *SmHMGR3* was up-regulated, but *SmHMGS, SmPMK,* and *SmMDC* were down-regulated as low as 34% of the control. The other genes contributing to MEP or MVA pathways remained nearly at the same levels. These results proved that overexpression of *SmMYB9b*-activated MEP pathway rather than MVA pathway in danshen hairy roots.

Discussion

In the phylogeny tree constructed here, SmMYB9b was clustered with six danshen MYBs, SmMYB9, SmMYB98, SmMYB31, SmMYB63, SmMYB81, and SmMYB38, and six *Arabidopsis* members, AtMYB108, AtMYB78, AtMYB112, AtMYB2, AtMYB116, and AtMYB62 into S20. There were no functional studies of these SmMYBs. The six *Arabidopsis* S20 members were reported to be involved in stress responses and related to morphological architectures like AtMYB2 in activating ABA-inducible gene expression under drought stress and inhibiting the branching by suppressing the cytokinin production during plant senescence (Abe et al. 2003; Guo and Gan 2011) and AtMYB62 in regulating the plant architecture through modulating the phosphate starvation responses and gibberellic acid biosynthesis during nutrient stress (Devaiah et al. 2009). As described, *SmMYB9b* transcripts were most abundant in flowers. Overexpression of *SmMYB9b* led to high accumulation of tanshinones especially CT (8.7-fold of the control) (Table 1) which was identified as a phytoalexin against pathogen attack in *S. miltiorrhiza* (Chen and Chen 2000). As reported, triterpenoids were identified from danshen flowers (Liu et al. 2011) and noticeable content of monoterpenoids were produced in corolla and calyx of *Salvia sclarea* L. (Schmiderer et al. 2008), the same Lamiaceae plant as danshen, which indicated that there were terpenoids accumulating in danshen flowers. In this study, trace amount of CT, TA-I, and TA-IIA were also detected in blooming flowers and flower buds, although they were mainly stored up in roots (Table S4). Taken together, it was proposed that SmMYB9b presumably functions as an activator in regulating terpenoid biosynthesis through possibly responding to pathogen attack or other biotic and abiotic stresses in danshen flowers.

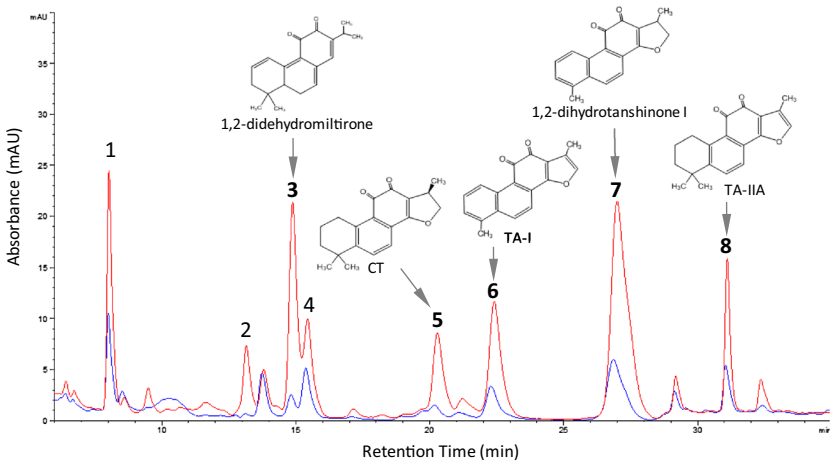

Fig. 4 Typical HPLC profile of tanshinones in SmMYB9b-OX hairy roots. *Line* in *red* represents the profile of SmMYB9b-OX hairy root line M11. *Line* in *blue* indicates that of the control (sample C3 as in Table S3). Eight principal compounds are labeled as 1–8 and identified as compound 487.5, 15,16-dihydrotanshinone I, 1,2-didehydromiltirone, methyltanshinonate, cryptotanshinone, tanshinone I, 1,2-dihydrotanshinone I, and tanshinone IIA, respectively. Crude sample concentration of control and M11 is 100.10 and 99.90 mg DW/ml, respectively (color figure online)

Table 1 Amount of diterpenoids and phenylpropanoids in SmMYB9b-OX hairy roots

Items	SmMYB9b-OX	Control
Tanshinones (mg/g DW)		
CT	0.29 ± 0.05 (8.7)	0.03 ± 0.01
TA-I	1.45 ± 0.28 (3.1)	0.48 ± 0.05
TA-IIA	0.42 ± 0.08 (0.9)	0.48 ± 0.21
TTA	2.16 ± 0.39 (2.2)	0.99 ± 0.25

Data of SmMYB9b-OX hairy roots represent mean ± SD of five independent biological replicates, those of control represent mean ± SD of eight (for tanshinones) independent biological replicates. Number in the parentheses indicates the folds or the percentage of the compound in SmMYB9b-OX hairy roots to that in the control. The sum of cryptotanshinone (CT), tanshinone I (TA-I), and tanshinone IIA (TA-IIA) is presented as total tanshinones (TTA)

On the other hand, the highest levels of differential gene expression were also found in terpenoid biosynthesis pathways and the transcription of a couple of genes responsible for ABA and GA biosynthesis was increased in *SmMYB9b* over expressing hairy roots. Especially, the gene encoding abscisic acid receptor PYR/PYL family (PYL) was up-regulated. In addition, exogenous application of ABA rather than GA and MeJA significantly activated the transcription of *SmMYB9b*. It appears that the role of SmMYB9b is closely related to ABA signaling. It seemed that overexpression of *SmMYB9b* could activate the terpenoid biosynthetic pathway, resulting in the biosynthesis of diterpenoids, such as tanshinones, and tetraterpenoids, such as carotenoids, switching on the ABA signaling, which in turn could stimulate the transcription of *SmMYB9b*. The facts that overexpression of *SmMYB9b* reduced the lateral hairy roots and hairy root growth, a similar phenotype variation to exogenous application of ABA to wild-type danshen hairy roots (Fig. S5), to some extent, also supported this speculation, as endogenous ABA levels play a critical role in lateral root formation (De Smet et al. 2003). As ABA signaling is the central regulator of abiotic stress response in plants (Danquah et al. 2014), the close relationship between SmMYB9b and ABA signaling indicated that SmMYB9b may play roles in responding to certain unfavorable biotic and abiotic signals in danshen flowers.

Table 2 Correlation analysis of SmMYB9b transcripts with biomass and tanshinone concentration from individual SmMYB9b-OX and control hairy roots

Correlation coefficiency	*SmMYB9b* Transcripts	Biomass	CT	TA-I	TA-IIA	TTA
SmMYB9b transcripts	1					
Biomass	−0.801*	1				
CT	0.828*	−0.940**	1			
TA-I	0.818*	−0.861*	0.977**	1		
TA-IIA	0.843*	−0.858*	0.978**	0.985**	1	
TTA	0.830*	−0.876*	0.985**	0.998**	0.992**	1

* Correlation is significant at the 0.05 level (two-tailed)

** Correlation is significant at the 0.01 level (two-tailed)

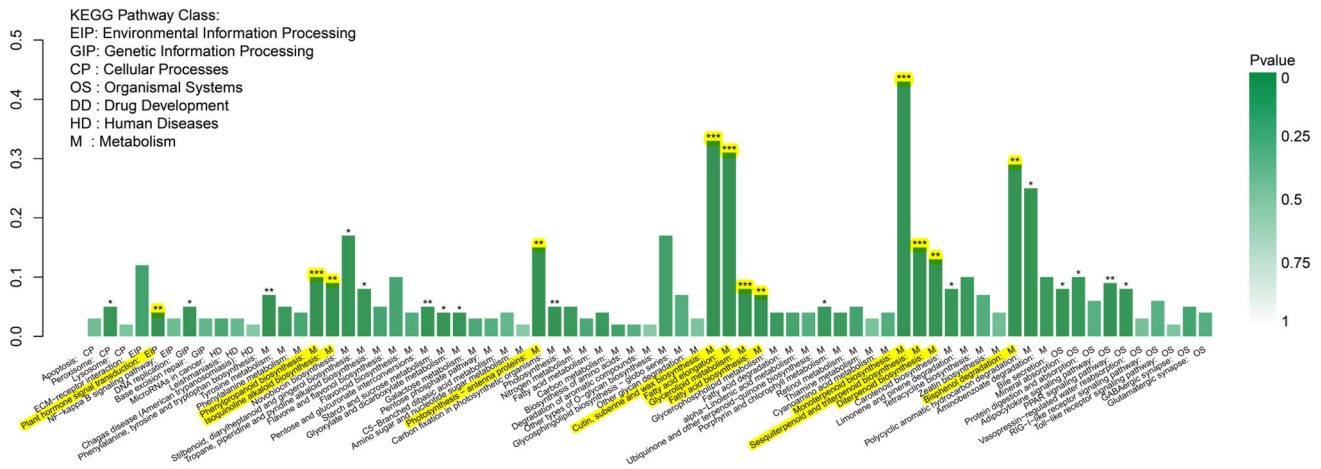

Fig. 5 KEGG pathway enrichment analysis with differential expression genes from de novo RNAseq data. *Asterisks* above the *columns* represent the *p* value. ***$p < 0.001$, **$p < 0.01$. *$p < 0.05$. Emphasized pathways were highlighted in *yellow* (color figure online)

Compared to ABA, the effects of GA and MeJA on *SmMYB9b* transcription was much weaker. It is possible that SmMYB9 played a weak role in GA and JA signaling, although exogenous application of MeJA also inhibited the wild-type hairy root growth and suppressed the lateral root formation as observed in the overexpression of *SmMYB9b* (Fig. S5); nevertheless, GA demonstrated little influence on hairy root growth. The transcription of *SmKSL2* was down-regulated in SmMYB9b-OX hairy roots. SmKSL2 was responsible for the biosynthesis of GAs, as reported, recombinant SmKSL2 enzyme combining with SmCPS5 resulted in the production of ent-kaurene, the diterpene precursor to GAs, in vitro (Gao et al. 2009). It was likely that SmMYB9b would negatively regulate GA signaling. To get a clearer idea of SmMYB9b's role in ABA-, GA-, and JA-signalings, it may be helpful to perform comparative analysis of the RNAseq data from SmMYB9b-OX hairy roots with those from ABA-, GA-, and MeJA-treated hairy roots in the future.

The high correlation between *SmMYB9b* transcripts and tanshinone concentration (Table 2) indicated that *SmMYB9b* acts as a direct activator in tanshinone biosynthesis. As indicated in KEGG pathway analysis, *GGPPS,* the terpenoid backbone biosynthesis gene, was up-regulated in SmMYB9b-OX hairy roots. As a more accurate gene expression quantification method (Sun et al. 2014), qRT-PCR was also carried out and the results demonstrated that overexpression of *SmMYB9b* promoted the transcription of two MEP upstream genes, *SmDXS2* and *SmDXR*, and two important branched terpenoid biosynthetic genes, *SmGGPPS* and *SmKSL1*. As reported, these genes were responsible for the biosynthesis of diterpenoids tanshinones (Wu et al. 2009; Kai et al. 2011; Zhou et al. 2012; Dai et al. 2012). In contrast, the transcription of the key enzyme encoding genes in MVA pathway, *SmHMGS* and *SmHMGR1* (Kai et al. 2011), was down-regulated, whereas *SmHMGR3* was up-regulated. The other MVA and MEP pathway genes were identified from the transcriptome and genome of *S. miltiorrhiza* (Ma et al. 2012; Yang et al. 2013) and some of them such as *SmPMK* and *SmMDC* were reported to positively correlate with tanshinone concentrations (Zhao et al. 2015). As shown in Fig. 6, the transcription of MVA pathway genes, *SmPMK*, *SmMDC*, and *SmFPPS,* were inhibited, while that of the MEP pathway gene, *SmMCT*, was increased in SmMYB9b-OX hairy roots. Taken together, it was likely that the improvement of tanshinone concentration was a result from the stimulated MEP pathway rather than MVA pathway in SmMYB9b-OX hairy roots.

It should be noted that we also quantitatively analyzed the phenylpropanoid metabolites, which showed that the concentration of salvianolic acids, the other bioactive components of danshen, was decreased to 70% of the control (Table S5), whereas the other phenylpropanoid derivatives anthocyanins and lignin were, respectively, increased to 1.3- and 1.1-fold of the control. The insignificant correlation between *SmMYB9b* transcripts and phenylpropanoid concentration (Table S6) implied that overexpression of *SmMYB9b* interfered with the phenylpropanoid metabolism possibly in an indirect manner.

In summary, an S20 R2R3-MYB, *SmMYB9b*, was isolated from danshen. The findings in this study proved that SmMYB9b could efficiently improve the tanshinone concentration. In addition, the simultaneous alteration of the growth and architecture of SmMYB9b-OX hairy roots as well as the transcriptomic variation hinted that SmMYB9b is presumably involved in the complicated regulatory network related to terpenoid biosynthesis, opening new

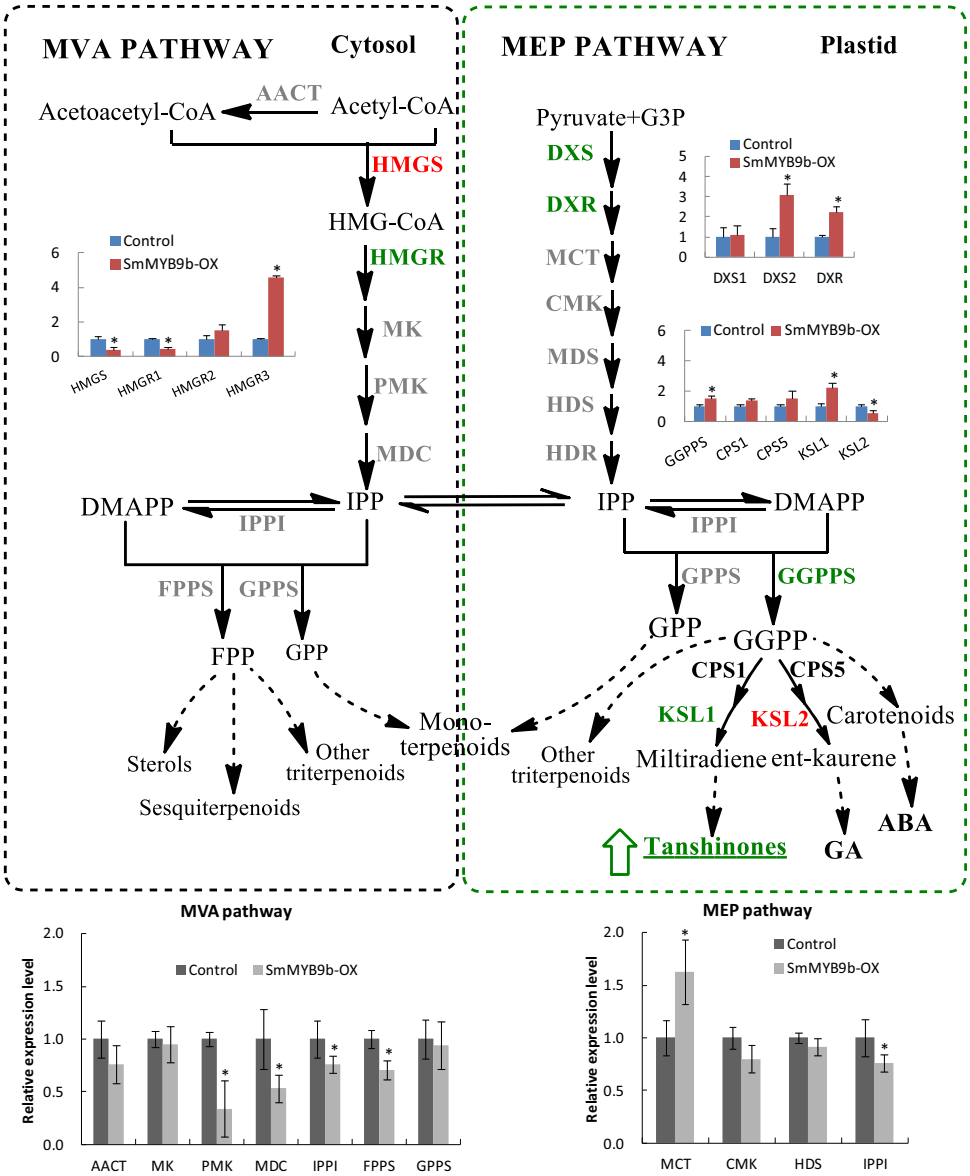

Fig. 6 Terpenoid pathway schematic and expression pattern of the biosynthetic genes in SmMYB9b-OX hairy roots. Graphs inside the pathway indicate the expression levels of the corresponding pathway genes. The *y* axis of the graphs presents relative expression level. Data represent mean ± SD from three to five independent biological replicates. One *asterisk* above the *columns* indicates that the difference is significant from the control ($p < 0.05$). Significantly up-regulated genes are indicated in *green*, down-regulated genes in *red*, unchanged genes in *black*. Enzymes without functional studies are shown in *grey* and their expression patterns are displayed below the schematic pathway. *Plain arrows* represent one enzymatic step; *dashed arrows* indicate unclear or multiple steps. *Half arrows* at both sides indicate that the compounds could be transported or transformed. A *green hollow up arrow* beside the compound indicates the concentration of the compound significantly increased (color figure online)

avenues for research on its function and for pathway engineering of terpenoid products such as tanshinones in the future.

Author contribution statement JXZ obtained SmMYB9b-OX hairy roots, performed phenylpropanoid quantification, the transcript assay, and drafted the corresponding part for the manuscript. LBZ cloned *SmMYB9b* gene, did its expression pattern assay, and drafted the corresponding part for the manuscript. XYZ helped perform the expression assay of pathway genes. JJZ quantified the concentration of tanshinones and salvianolic acids and performed hormone supplementary experiments. RHT helped to analyze the data of qPCR assay. LY did the LC–MS analysis. SJZ put forward the project, did hormone-induced gene expression, analyzed the data, and finished the manuscript. All authors approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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