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Tanshinone and salvianolic acid biosynthesis are regulated by *SmMYB98* in *Salvia miltiorrhiza* hairy roots

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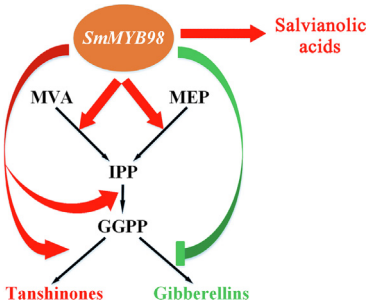
HIGHLIGHTS

- *SmMYB98* was predominantly expressed in the lateral roots of *Salvia miltiorrhiza*.
- Tanshinones and salvianolic acids were improved in the *SmMYB98-OE* hairy roots.
- Tanshinone and salvianolic acid contents in *SmMYB98-KO* lines were reduced.
- *SmMYB98* regulates the expression of tanshinones and salvianolic acids biosynthetic genes.
- *SmMYB98* negatively regulates the biosynthesis of gibberellins in *S. miltiorrhiza*.

GRAPHICAL ABSTRACT



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ABSTRACT

Salvia miltiorrhiza Bunge is an herb rich in bioactive tanshinone and salvianolic acid compounds. It is primarily used as an effective medicine for treating cardiovascular and cerebrovascular diseases. Liposoluble tanshinones and water-soluble phenolic acids are a series of terpenoids and phenolic compounds, respectively. However, the regulation mechanism for the simultaneous promotion of tanshinone and salvianolic acid biosynthesis remains unclear. This study identified a R2R3-MYB subgroup 20 transcription factor (TF), *SmMYB98*, which was predominantly expressed in *S. miltiorrhiza* lateral roots. The accumulation of major bioactive metabolites, tanshinones, and salvianolic acids, was improved in *SmMYB98* overexpression (OE) hairy root lines, but reduced in *SmMYB98* knockout (KO) lines. The qRT-PCR analysis revealed that the transcriptional expression levels of tanshinone and salvianolic acid biosynthesis genes

Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonate; MEP, 2-C-methyl-D-erythritol 4-phosphate; AACT, acetoacetyl-CoA thiolase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, mevalonate diphosphate decarboxylase; IPPI, isopentenyl diphosphate isomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, MEP cytidyl-transferase; CMK, 4-(cytidine5-diphospho)-2-C-methylerythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, hydroxy-methybutenyl-4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; TAT, tyrosine aminotransferase; HPPR, 4-hydroxyphenylpyruvate reductase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; RAS, rosmarinic acid synthase; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-pyrophosphate; G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMB-PP, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; CPP, copalyldiphesphate; ent-CPP, ent-Copalyldiphesphate.

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were upregulated by *SmMYB98-OE* and downregulated by *SmMYB98-KO*. Dual-Luciferase (Dual-LUC) assays demonstrated that *SmMYB98* significantly activated the transcription of *SmGGPPS1*, *SmPAL1*, and *SmRAS1*. These results suggest that *SmMYB98-OE* can promote tanshinone and salvianolic acid production. The present findings illustrate the exploitation of R2R3-MYB in terpenoid and phenolic biosynthesis, as well as provide a feasible strategy for improving tanshinone and salvianolic acid contents by MYB proteins in *S. miltiorrhiza*.

Introduction

Danshen (*Salvia miltiorrhiza* Bunge) is a valuable and well-known traditional Chinese medicinal herb primarily used as an effective medicine for treating cardiovascular and cerebrovascular diseases [1–6]. Liposoluble tanshinones, including tanshinone I (TI), tanshinone IIA (TIIA), cryptotanshinone (CT), and dihydrotanshinone (DT), are the major bioactive terpenoids of *S. miltiorrhiza* (Fig. S1) [5,7–10]. Water-soluble salvianolic acids, including rosmarinic acid (RA), salvianolic acid B (SAB), salvianolic acid A (SAA), and caffeic acid (CA), are the major bioactive phenolic compounds of *S. miltiorrhiza* (Fig. S1) [5,7–10]. Due to the increasing clinical demand for *S. miltiorrhiza*, it is necessary to apply novel metabolic engineering strategies to improve and enhance the biosynthesis of bioactive compounds of this plant [1,2,11].

Tanshinones exhibit a variety of biological activities in clinical applications, including antimicrobial, antioxidant, anti-cancer, anti-inflammation, and anti-allergic effects [1,12,13]. Tanshinones are a type of diterpenoid that are biosynthesized in three stages (Fig. 1) [14,15]. First, common terpenoid precursors (i.e., IPP and DMAPP) are synthesized from two different pathways (i.e., the MVA pathway localized in the cytosol and MEP pathway that occurs in plastids) [2,14,16]. Next, geranylgeranyl diphosphate synthase (GGPPS) catalyzes the biosynthesis of the universal diterpenoid precursor, GGPP [8,14,17]. Finally, tanshinones are synthesized by three identified synthases (i.e., copalyl diphosphate synthase 1 (CPS1), kaurene synthase-like 1 (KSL1), and miltiradiene oxidase (CYP76AH1)) and another unidentified enzyme (Fig. 1) [15,18,19]. As a diterpenoid phytohormone, gibberellins (GAs) are also synthesized from GGPP and then catalyzed by four synthases (i.e., copalyl diphosphate synthase 5(CPS5), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO)). Bioactive GAs are produced by the catalysis of GA_{20}-oxidase (GA_{20}ox) and GA_3-oxidase (GA_3ox), and

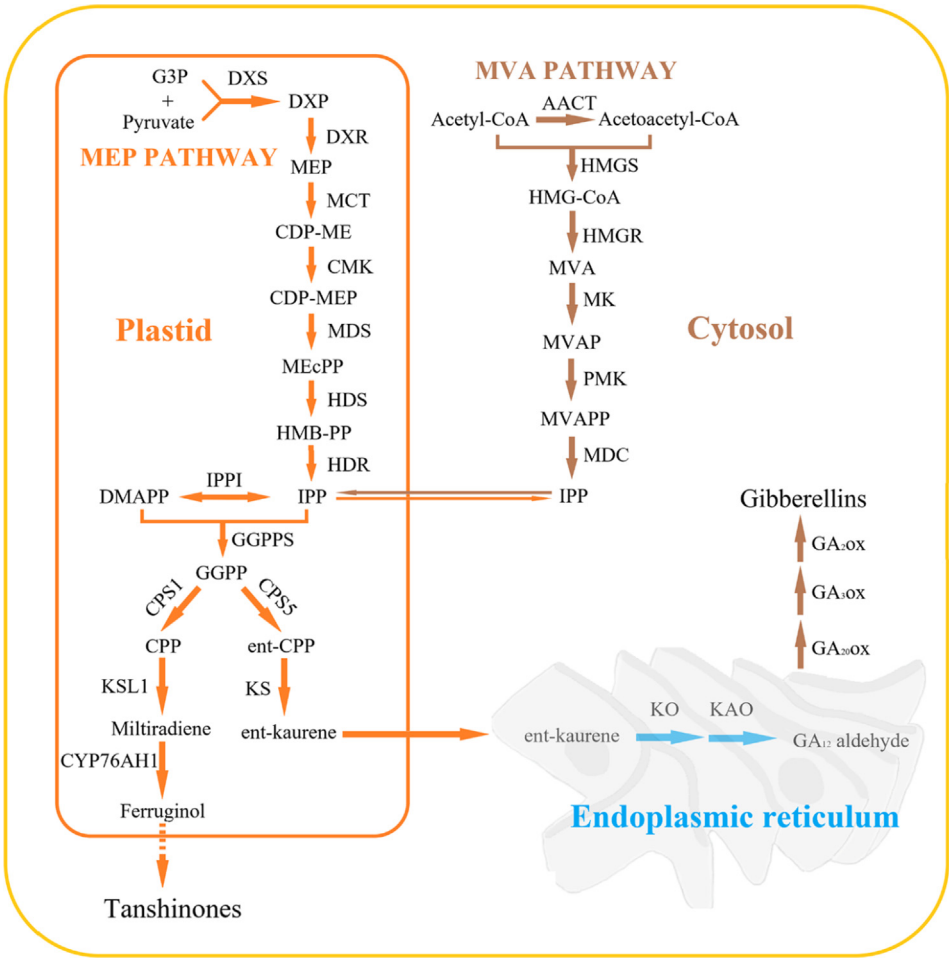

Fig. 1. Biosynthetic pathway of tanshinones and GAs in *S. miltiorrhiza*. MVA pathway, mevalonate pathway; MEP pathway, 2-*C*-methyl-D-erythritol 4-phosphate pathway. Abbreviations for synthetases and compounds refer to the Abbreviations table and Introduction text.

deactivated by GA_2-oxidase (GA_2ox) in *S. miltiorrhiza* (Fig. 1) [20,21]. Salvianolic acids, another main bioactive compound found in *S. miltiorrhiza*, are biosynthesized through the phenylpropanoid and tyrosine-derived pathways. The biosynthetic genes involved in the salvianolic acid pathway, including *TAT*, *HPPR*, *PAL*, *C4H*, *4CL*, *RAS*, and *CYP98A14*, have been partially illustrated (Fig. 2) [10].

MYB transcription factor (TF) proteins are a large plant TF family and generally contain three types of MYB domains (i.e., R1, R2, and R3), which consist of 52 amino acid residues [22,23]. Most MYB proteins are R2R3-MYB TFs. Their N-terminal contains a highly conserved R2R3-MYB domain, and the C-terminal contains an activation or inhibition domain [22–24]. According to the characteristics of these two conserved domains, R2R3-MYB TFs have been classified into different subgroups with various functions [22,23]. Compared to *Arabidopsis thaliana* R2R3-MYB TFs, 110 R2R3-MYB proteins have been identified in *S. miltiorrhiza*, which were further divided into 37 subgroups [25,26]. Four S20 subgroup R2R3-MYB genes (i.e., *SmMYB9*, *SmMYB31*, *SmMYB81*, and *SmMYB98*) were predicted to participate in terpenoid biosynthesis [25]. Consistent with this deduction, *SmMYB9b* has been identified and verified to be an activator of tanshinone biosynthesis [27]. Furthermore, *AtMYB62* in *A. thaliana* has been reported to be involved in GA biosynthesis induced by Pi starvation and *AtMYB62* overexpression (OE), which can suppress the expression of early GA biosynthetic genes to modulate GA biosynthesis [28]. However, no studies have investigated S20 R2R3-MYB TFs that simultaneously regulate tanshinones and salvianolic acids in *S. miltiorrhiza*.

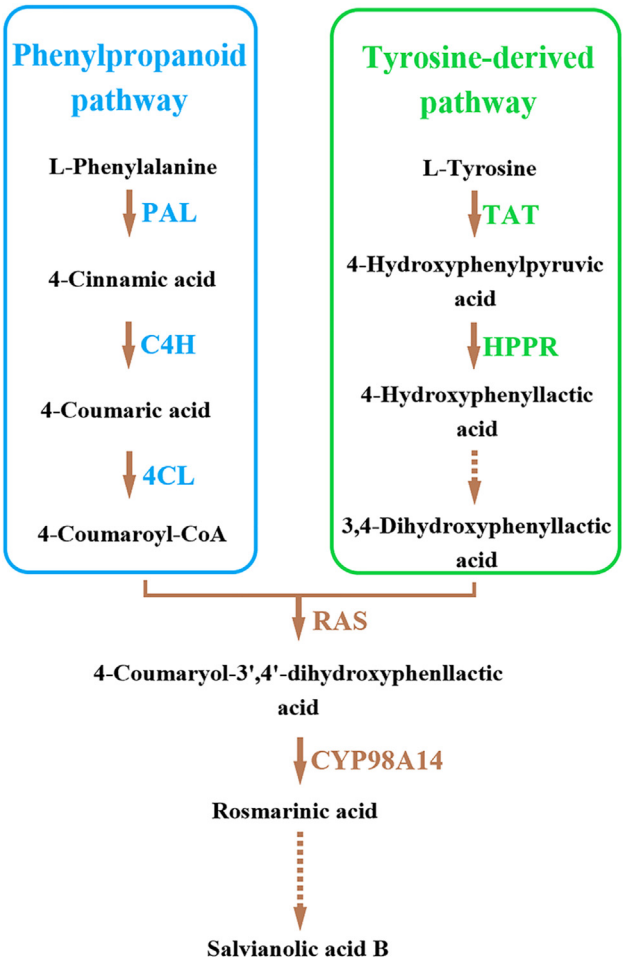

Fig. 2. Salvianolic acid biosynthetic pathway in *S. miltiorrhiza*. Abbreviations for synthetases and compounds refer to the Abbreviations table and Introduction text.

In this study, a R2R3-MYB S20 subgroup member, *SmMYB98*, was isolated from *S. miltiorrhiza*, and its function was investigated. Results revealed that *SmMYB98* could enhance tanshinone and salvianolic acid accumulation, as well as decrease GA biosynthesis. *SmMYB98* also changed the architecture of the hairy roots. These findings indicate that *SmMYB98* is capable of simultaneously promoting tanshinone and salvianolic acid biosynthesis in *S. miltiorrhiza*.

Materials and methods

Plant materials

S. miltiorrhiza plants were collected and cultivated in a greenhouse as previously described [1,16,29]. Five different *S. miltiorrhiza* tissues, including the taproots, lateral roots, stems, leaves, and flowers, were collected from one-year-old *S. miltiorrhiza* plants for RNA isolation to analyze the tissue expression patterns of different genes. *S. miltiorrhiza* plantlets used for hairy root transformation mediated by *Agrobacterium* were cultivated in solid Murashige and Skoog (MS) medium under a 16/8 h light/dark photoperiod at 25 °C [30]. Different *S. miltiorrhiza* hairy root lines were cultured in 150 mL conical flasks with 50 mL 1/2 MS liquid medium and placed in a shaker under total darkness at 25 °C [30]. *Nicotiana benthamiana* were planted in pots, placed in a growth chamber, and subsequently used for subcellular localization and Dual-Luciferase (Dual-LUC) experiments [31].

Bioinformatic analysis

SmMYB98 full-length cDNA was amplified from the *S. miltiorrhiza* cDNA library using *SmMYB98* gene-specific primers (Table S1). The phylogenetic tree was constructed using the *SmMYB98* gene and 18 members of the R2R3-MYB S20 subgroup from different plants using the neighbor-joining method and bootstrap analyses with 1000 replicates, which was performed using MEGA v.5 software, to analyze and evaluate the accuracy of the phylogeny [32]. The protein sequences from *S. miltiorrhiza* (SmMYB98, AGN52122.1; SmMYB9b, AGG09670.1; SmMYB31, AGN52055.1; SmMYB81, AGN52105.1), *A. thaliana* (AtMYB2, OAP10937.1; AtMYB62, OAP19074.1; AtMYB78, OAO94036.1; AtMYB108, AEE74402.1; AtMYB112, OAP18778.1; AtMYB116, OAP18163.1), *Camelina sativa* (CsMYB108, XP_010506747.1), *Brassica napus* (BnMYB2, XP_013691919.1) *Raphanussativus* (RsMYB108, XP_018438809.1), *Capsella rubella* (CrMYB62, XP_006301020.1), *Eutrema salsugineum* (EsMYB62, XP_006391151.1), *Sesamum indicum* (SiMYB62, XP_011076599.1), *Nicotiana attenuata* (NaMYB108, XP_019256818.1), *Artemisia annua* (AaMYB62, XP_021976512.1), and *Oryza sativa* (OsMYB1, XP_015633684.1) were collected from the NCBI database. *SmMYB98* and four R2R3 MYB S20 subfamily genes from different species were aligned using ClustalW [33].

Plant RNA isolation and qRT-PCR analysis

Total RNA of the five *S. miltiorrhiza* tissues and two different hairy root lines (i.e., *SmMYB98-OE* and *SmMYB98-KO*) was isolated using a plant RNA prep pure kit (Tiangen Biotech Co., Ltd., Beijing, China) [2,30]. cDNA of each sample was synthesized and qRT-PCR analyses were performed as previously described [5]. The *S. miltiorrhiza actin* gene was used as the internal reference gene. All primer sequences used for qRT-PCR analyses are provided (Table S1). The relative expression values of different genes from different samples were calculated using the $2^{-\Delta\Delta Ct}$ method [2,30].

Plant expression vector construction

The full-length *SmMYB98* ORF was inserted into *pHB*-yellow fluorescent protein (YFP) to obtain the *pHB-SmMYB98-YFP* construct, which was used to transform *S. miltiorrhiza* to generate hairy roots and transiently transform *N. benthamiana* leaves. The *pHB-YFP* empty vector without the *SmMYB98* gene was used as the control (Fig. S2A). For *SmMYB98* knockout (KO) vector construction, the potential gene editing sites of the *SmMYB98* gene sequence were analyzed using Optimized CRISPR Design (http://crispr.dbcls.jp/). Then, a pair of complementary oligos were synthesized and ligated to the CRISPR/Cas9 system expression protein to combine sgRNA and hSpCas9. Thus, hSpCas9 was driven by the CaMV 35S promoter and *SmMYB98* sgRNA was driven by the *AtU6* promoter (Fig. S2B) [34,35]. Then, the expression cassette was inserted into the linearized plant expression vector, *pCAMBIA1300*, for infecting *S. miltiorrhiza* leaves to generate hairy roots (Fig. S2B). The *pCAMBIA1300* empty vector without the sgRNA sequence was used as the control (Fig. S2B) [34,35].

Subcellular localization analysis

In order to investigate the subcellular localization of the SmMYB98 protein, the *pHB-SmMYB98-YFP* and *pHB-YFP* constructs were transferred into the GV3101 *Agrobacterium tumefaciens* strain. *pHB-YFP* without *SmMYB98* was used as the negative control. The two GV3101 strains were transiently transformed into the epidermal cells of two symmetrical parts of the same *N. benthamiana* leaf [29]. YFP signals of infected *N. benthamiana* leaves were detected using a confocal microscopy (Leica Microsystems, Wetzlar, Germany) after *A. tumefaciens* infection for 48 h. Three hours before observation, DAPI solution was injected to stain the nuclei in the epidermal cells of infected *N. benthamiana* leaves.

Generation of SmMYB98 transgenic S. miltiorrhiza hairy roots

All recombinants were introduced into the disarmed C58C1 *A. tumefaciens* strain, which harbored the *A. rhizogenes* Ri plasmid, pRiA4, and infected *S. miltiorrhiza* leaves to generate different transgenic hairy root lines as previously described [1]. These different potential hairy root candidates were screened on a 1/2 MS media plate. Positive transgenic hairy roots were selected via PCR amplification. Primers were designed to cover the gene and partial vector sequences for *SmMYB98* amplification. Then, positive transgenic hairy roots were further cultured on 1/2 MS liquid medium for two months.

Dual-LUC assays

Reporter constructs (*pSmDXS2*::*fLUC*, *pSmDXR*::*fLUC*, *pSmHMGR1*::*fLUC*, *pSmGGPPS1*::*fLUC*, *pSmCPS1*::*fLUC*, *pSmKSL1*::*fLUC*, *pSmCYP76AH1*::*fLUC*, *pSmPAL1*::*fLUC*, *pSmC4H1*::*fLUC*, *pSm4CL1*::*fLUC*, *pSmTAT1*::*fLUC*, *pSmHPPR1*::*fLUC*, *pSmRAS1*::*fLUC* and *pSmCYP98A14*::*fLUC*) were obtained by inserting the promoters of seven tanshinone biosynthetic genes and seven salvianolic acid biosynthetic genes into the *pGreenII 0800-LUC* vector to drive the expression of the firefly luciferase gene [31,36]. The assembled vectors were transformed into the *A. tumefaciens* strain, GV3101. Renilla luciferase was driven by the CaMV 35S promoter and used as the internal control. The GV3101 strains harboring either *pHB-SmMYB98-YFP* or *pHB-YFP* were used as the effector and control, respectively. Infiltration and detection were performed as previously described with minor modifications [31,36]. The mixed suspension of the reporter and effector strains was infiltrated into *N. benthamiana* leaves; negative controls were infiltrated into the opposite position of the same leaves. Leaves were collected after being kept in total darkness for two days. Dual-LUC assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions.

Determination of tanshinone and phenolic acid contents

Different hairy root lines were harvested after culturing on 1/2 MS liquid medium for two months and dried in a freeze-drying machine for ~24 h. For tanshinone measurements, ~50 mg dried hairy root powder was extracted with 16 mL methanol/dichloromethane (3:1, v/v) and sonicated for 1 h. For phenolic acid measurements, ~100 mg dried hairy root powder was extracted with 10 mL ethanol/water (4:1, v/v) and sonicated for 30 min. High performance liquid chromatography (HPLC) analysis of tanshinone and phenolic acid extracts was performed using an Agilent 1260 detector equipped with a reversed-phase C18 symmetry column (Agilent Technologies, Palo Alto, CA, USA) as previously described [5,11,13]. Tanshinone detection conditions were as follows: mobile phase, acetonitrile: water (65:35, v/v); column temperature, 30 °C; detection wavelength, 220 nm [5,11]. Phenolic acids detection conditions were as follows: mobile phase, acetonitrile: water (3:7, v/v); column temperature, 35 °C; detection wavelength, 281 nm [5,11]. The detection and quantification of four tanshinones (i.e., TI, CT, TIIA, and DT) and four phenolic acids (i.e., RA, SAB, CA, and SAA) were conducted by comparing the retention times and standard curves. These eight compounds were qualitatively analyzed in hairy root cultures by liquid chromatography-tandem mass spectrometry (LC-MS) as previously described (Fig. S1) [5,11]. Total tanshinones and total phenolic acids represented a total of four detected compounds. The tanshinone and phenolic acid standards were used for comparative quantification of the compounds found in the plant samples (Aladdin, China) as previously described [8].

Extraction and detection of GAs in S. miltiorrhiza hairy roots

Due to the low GA contents in plants, it is difficult to extract and detect GAs. Therefore, the extraction and detection of GAs in *S. miltiorrhiza* hairy roots were performed by the Suzhou Keming Biotechnology Co., Ltd. (Suzhou, China). The extraction and detection methods were optimized and modified according to the reported literature [37,38]. First, different hairy root samples were ground to a powder in the presence of liquid nitrogen. Different powders (0.2 g) were extracted twice at 4 °C for 12 h with 1 mL pre-cooling 80% methanol. After centrifugation, two supernatants were combined and dried at 40 °C with nitrogen. Then, 0.5 mL petroleum ether was added to the extract three times; the upper phase was discarded. The pH of the lower phase was adjusted to 2.8 with 2 M citric acid and extracted with an equal volume of ethyl acetate three times. Subsequently, the organic phase was dried with nitrogen and 0.5 mL methanol was added for detection. GA detection conditions were as follows: mobile phase, methanol: acetic acid (3:7, v/v); column temperature, 30 °C; detection wavelength, 254 nm.

Statistical analyses

All experiments were conducted with at least three biological replicates. The data are presented as the mean ± standard deviation (SD). In order to compare the differences between different groups, a paired two-tailed Student's *t*-test was conducted with a significance threshold of $p < 0.05$.

Results

Isolation and characterization of SmMYB98

Previously, 110 R2R3-MYB proteins were identified in *S. miltiorrhiza* and four S20 subgroup R2R3-MYB genes (i.e., *SmMYB9*, *SmMYB31*, *SmMYB81*, and *SmMYB98*) were predicted to participate in terpenoid biosynthesis [25]. To further study the function of the S20 R2R3-MYB member, the full length of *SmMYB98* was isolated from cDNA and genomic DNA. *SmMYB98* contains a full-length 699 bp ORF that encodes a 232 amino acid protein with a calculated molecular mass of 27.08 kD and *pH* of 8.33. The genomic sequence of *SmMYB98* is 763 bp and contains one intron. The phylogenetic analysis of 19 *SmMYB98*-related genes from different species placed *SmMYB98* close to *SmMYB31*, *AaMYB62*, and *SiMYB62* (Fig. 3A). Furthermore, SmMYB98 and the other four reported S20 R2R3-MYB subgroup proteins (i.e., SmMYB9b, AtMYB62, AtMYB2, and OsMYB1) shared the conserved R2 domain, R3 domain, and WxPRL motif, which are characteristic domains of S20 R2R3-MYB subgroup proteins (Fig. 3B). These results indicate that *SmMYB98* has a very conservative domain [27,28,39].

Expression patterns of SmMYB98

In order to study the tissue expression patterns of *SmMYB98*, five different tissues from one-year-old *S. miltiorrhiza* were collected and analyzed by qRT-PCR. *SmMYB98* was highly expressed in the lateral roots and exhibited relatively low expression levels in the leaves (Fig. 4A). The tissue expression patterns of three tanshinone biosynthesis genes, including *SmCPS1*, *SmKSL1*, and *SmCYP76AH1*, were specifically expressed in the roots (i.e., taproots and lateral roots) (Fig. 4B). These results indicated that the expression patterns of *SmMYB98* were not tissue-specific, but were mainly expressed in the lateral roots where tanshinones accumulate.

To experimentally verify that *SmMYB98* encoded a TF, transient transformation assays in *N. benthamiana* leaves were conducted to study its subcellular localization. *SmMYB98* was inserted into the *pHB-YFP*, a *pHB* vector fused with the *YFP* reporter gene under the 35S promoter, to produce the *pHB-SmMYB98-YFP* construct. Next, the constructed *SmMYB98* plant expression vector and control vector, *pHB-YFP*, were transformed into GV3101 strains and transiently expressed in *N. benthamiana* leaves. Results revealed that the *SmMYB98* fluorescent signal was only detected in the nucleus of *N. benthamiana* leaves (Fig. 4C). Overall, the expression patterns of *SmMYB98* were consistent with a TF.

Generation of the SmMYB98-OE and SmMYB98-KO hairy root lines

To detect the role of *SmMYB98* in the hairy roots of *S. miltiorrhiza*, the recombinant OE vector, *pHB-SmMYB98-YFP*, was introduced into the disarmed C58C1 strain containing the pRiA4 plasmid. Then, *S. miltiorrhiza* explants were infected with the C58C1 strain containing *pHB-SmMYB98-YFP*; *pHB-YFP* was used as the control. Fresh and independent hairy roots could differentiate from the explants of infected leaves and stems (Fig. S3). The positive transgenic hairy root lines carrying *SmMYB98* were identified by PCR. The positive rate of 50 hairy root lines was 38.0% (Fig. S4). Five positive *pHB-YFP* hairy roots with a positive rate of 29.4% among 17 hairy root lines were also obtained (Fig. S5). qRT-PCR revealed that *SmMYB98* was expressed 1.13 to 456.52-fold higher in the *SmMYB98-OE* lines compared to the control (Fig. 5A). Three *SmMYB98-OE* lines with the highest expression levels were selected for further analysis.

CRISPR/Cas9 is a convenient and efficient genome editing technology that has been widely used in biological research. Two recent studies found that the rosmarinic acid synthase gene, *SmRAS*, which is involved in phenolic acid biosynthesis and the committed diterpene synthase gene, *SmCPS1*, which is involved in tanshinone biosynthesis, could be precisely and effectively knocked out using CRISPR/Cas9 in *S. miltiorrhiza* [34,35]. In this study, sgRNA of *SmMYB98* was inserted into a modified *pCAMBIA1300* vector to obtain a CRISPR-Cas9-KO vector. Then, the recombinant vector, *pCAMBIA1300-CRISPR/Cas9-SmMYB98sgRNA*, was introduced into C58C1; the *pCAMBIA1300* empty vector was used as the control. The positive lines carrying *sgRNA* of the *SmMYB98* gene were identified by PCR. The positive rate of 95 hairy root lines was 31.6% (Fig. S6). Then, the *SmMYB98* gene was amplified for DNA sequencing (Fig. S6). According to the DNA sequencing results, seven homozygous *SmMYB98-KO* hairy root lines were obtained (Fig. 5B, Fig. S7). Among the 14 hairy root lines, five positive *pCAMBIA1300* hairy root lines with a positive rate of 35.7% were also obtained (Fig. S8). qRT-PCR revealed that the expression

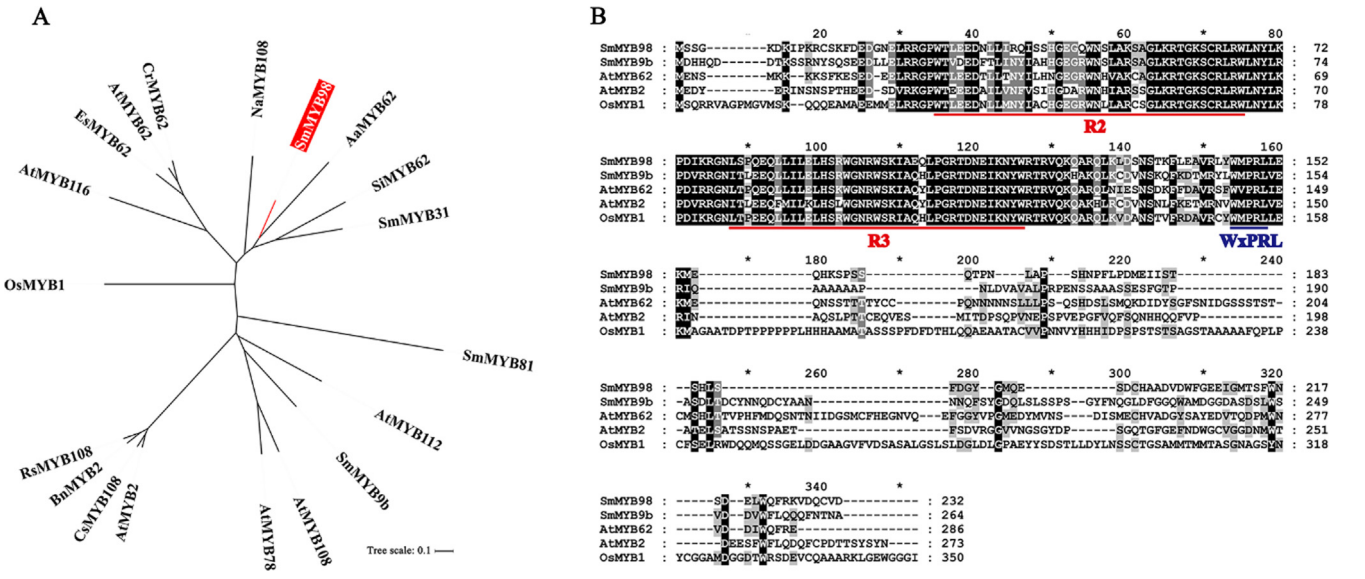

Fig. 3. Comparative analysis of *SmMYB98* and other related sequences. (A) Phylogenetic analysis of the *SmMYB98* gene and 18 members of the R2R3-MYB S20 subgroup from different plants. (B) Protein sequence alignment of SmMYB98 and four R2R3-MYB S20 subgroup proteins from different plants. The conserved R2 and R3 domains are underlined in red; the WxPRL core sequence is underlined in blue.

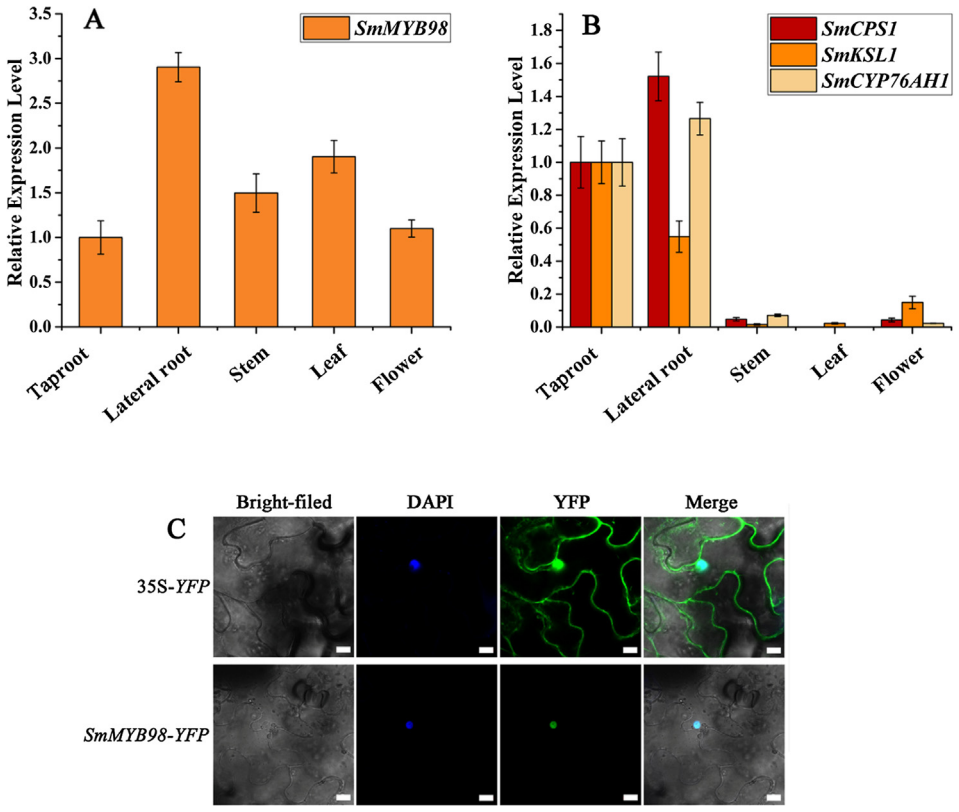

Fig. 4. Tissue expression and subcellular localization patterns of *SmMYB98*. (A, B) The differential transcriptional expression levels of the *SmMYB98*, *SmCPS1*, *SmKSL1*, and *SmCYP76AH1* genes from five different *S. miltiorrhiza* tissues (i.e., taproots, lateral roots, stems, leaves, and flowers) were detected by qRT-PCR. The transcriptional expression level of each gene in the taproots was set to 1. (C) Subcellular localization of 35S:SmMYB98-YFP (scale bars: 10 μm) and 35S:YFP (scale bars: 20 μm) in *N. benthamiana* leaf epidermal cells.

of *SmMYB98* in the *SmMYB98-KO* lines was 1.7- to 4.2-fold lower compared to the control (Fig. 5C). Three *SmMYB98-KO* lines with the lowest expression levels were selected for further analysis.

Metabolite analysis of the SmMYB98-OE and SmMYB98-KO hairy root lines

After culturing in 1/2 MS liquid medium for two months, the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines were harvested to observe the growth phenotypes and detection of metabolites. The phenotypes of the *SmMYB98-OE* hairy root lines were dark red compared to the control (Fig. 6A), while no obvious color change was observed in the *SmMYB98-KO* hairy root lines (Fig. 6B). It was previously reported that R2R3-MYB S20 members are involved in terpenoid biosynthesis. Tanshinones are the main active diterpenoid of *S. miltiorrhiza* roots and four types of tanshinones, including DT, CT, TI, and TIIA, were examined using HPLC in the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines. Results revealed that the total tanshinone contents were significantly improved and ranged from 7.7 to 15.4 mg/g DW in the *SmMYB98-OE* hairy root lines (Fig. 6C). Among them, *SmMYB98-OE-18* exhibited the highest *SmMYB98* expression levels and accumulated the greatest total tanshinone content, which was 3-fold higher compared to the control. The *SmMYB98-KO* lines accumulated lower total tanshinone contents with an average content of 4.4 mg/g DW (Fig. 6E). These results demonstrate that *SmMYB98* could positively regulate tanshinone biosynthesis in *SmMYB98* hairy roots.

Salvianolic acids are another main bioactive compound found in *S. miltiorrhiza* roots and four types of salvianolic acids, including SAA, SAB, CA, and RA, were examined using HPLC in the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines. Results revealed that the total salvianolic acid content was slightly upregulated from 15.4 to 20.9 mg/g DW in the *SmMYB98-OE* hairy root lines (Fig. 6D). The total salvianolic acid content of *SmMYB98-OE-18* was the highest, ~1.5-fold higher compared to the control. Moreover, the total salvianolic acid content was lower in the *SmMYB98-KO* lines (Fig. 6F). These results demonstrate that *SmMYB98* could positively regulate salvianolic acid biosynthesis in *SmMYB98* hairy roots.

Transcription level analysis of the SmMYB98-OE and SmMYB98-KO hairy root lines

In order to further explore the regulation mechanism of *SmMYB98* on tanshinone biosynthesis, the transcriptional expression levels of tanshinone biosynthetic genes in the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines were detected by qRT-PCR. Compared to the control, the transcription levels of most genes in the tanshinone biosynthesis pathway increased in all *SmMYB98-OE* transgenic hairy root lines. The most obvious changes were detected in the transcription levels of *SmDXS2* in the MEP pathway and *SmCPS1* in the downstream pathway, which were 4.5- and 4.8-fold higher compared to the control, respectively (Fig. 7). Additionally, the expression levels of *SmGGPPS1*, *SmKSL1*, and *SmCYP76AH1* in the *SmMYB98-OE* hairy root lines were >2.0-fold higher compared to the control (Fig. 7A and C). However, in the *SmMYB98-KO* hairy root lines, only the expression levels of *SmKSL1* were slightly downregulated (Fig. 7C). These results further suggest that *SmMYB98* could be a positive regulator of tanshinone biosynthesis.

Salvianolic acid compounds are biosynthesized through the phenylpropanoid and tyrosine-derived pathways (Fig. 2). Like tan-

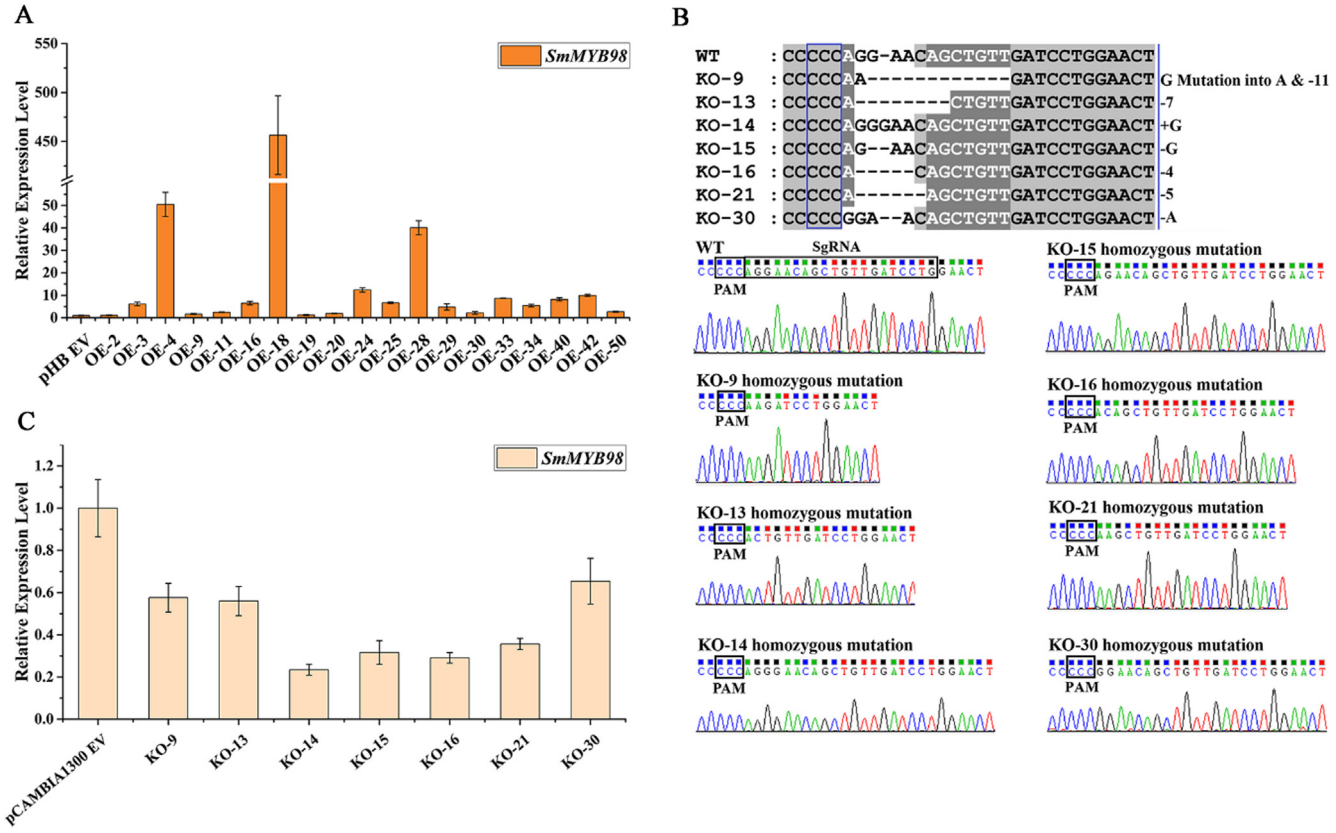

Fig. 5. Generation of the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines. (A, C) The transcriptional expression levels of *SmMYB98* in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by qRT-PCR. The average transcriptional expression level of *SmMYB98* in the two control hairy root lines was set to 1. The *S. miltiorrhiza actin* gene was used as the internal reference gene. Error bars represent the SD of three technical replicates. (B) Genomic *SmMYB98* DNA sequences from different *SmMYB98-KO* transgenic hairy root lines were detected by DNA sequencing. The original sequence of *SmMYB98* is displayed at the top; the PAM (GGG) area is highlighted in the blue box. Detailed DNA insertions and point mutations of the *SmMYB98* sequence in different *SmMYB98-KO* transgenic hairy root lines are presented below the original sequence.

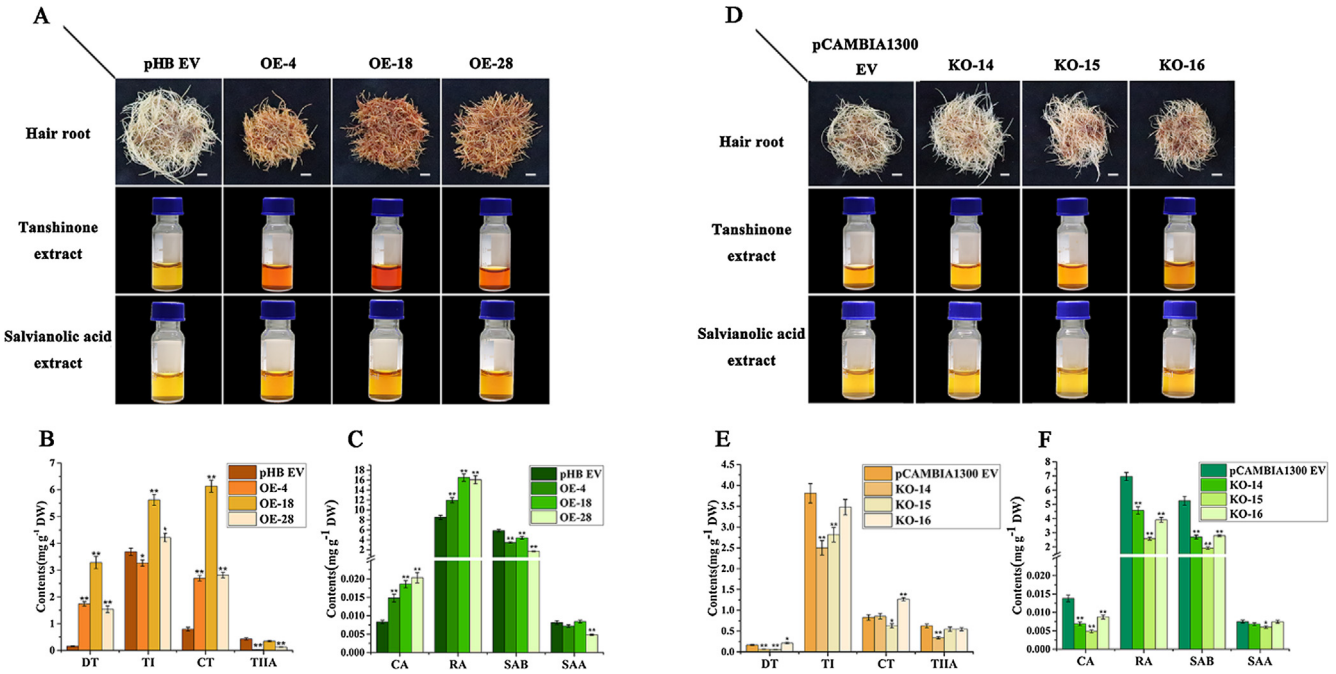

Fig. 6. Analysis of the tanshinones and salvianolic acids in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines. (A, D) The phenotype and tanshinone and salvianolic acid extracts of the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines (scale bars: 1 cm). (B, E) The contents of four tanshinones in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by HPLC. Error bars represent the SD of three technical replicates. (C, F) The contents of four salvianolic acids in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by HPLC. Error bars represent the SD of three technical replicates. TI, tanshinone I; TIIA, tanshinone IIA; CT, cryptotanshinone; DT, dihydrotanshinone; RA, rosmarinic acid; SAB, salvianolic acid B; SAA, salvianolic acid A; CA, caffeic acid.

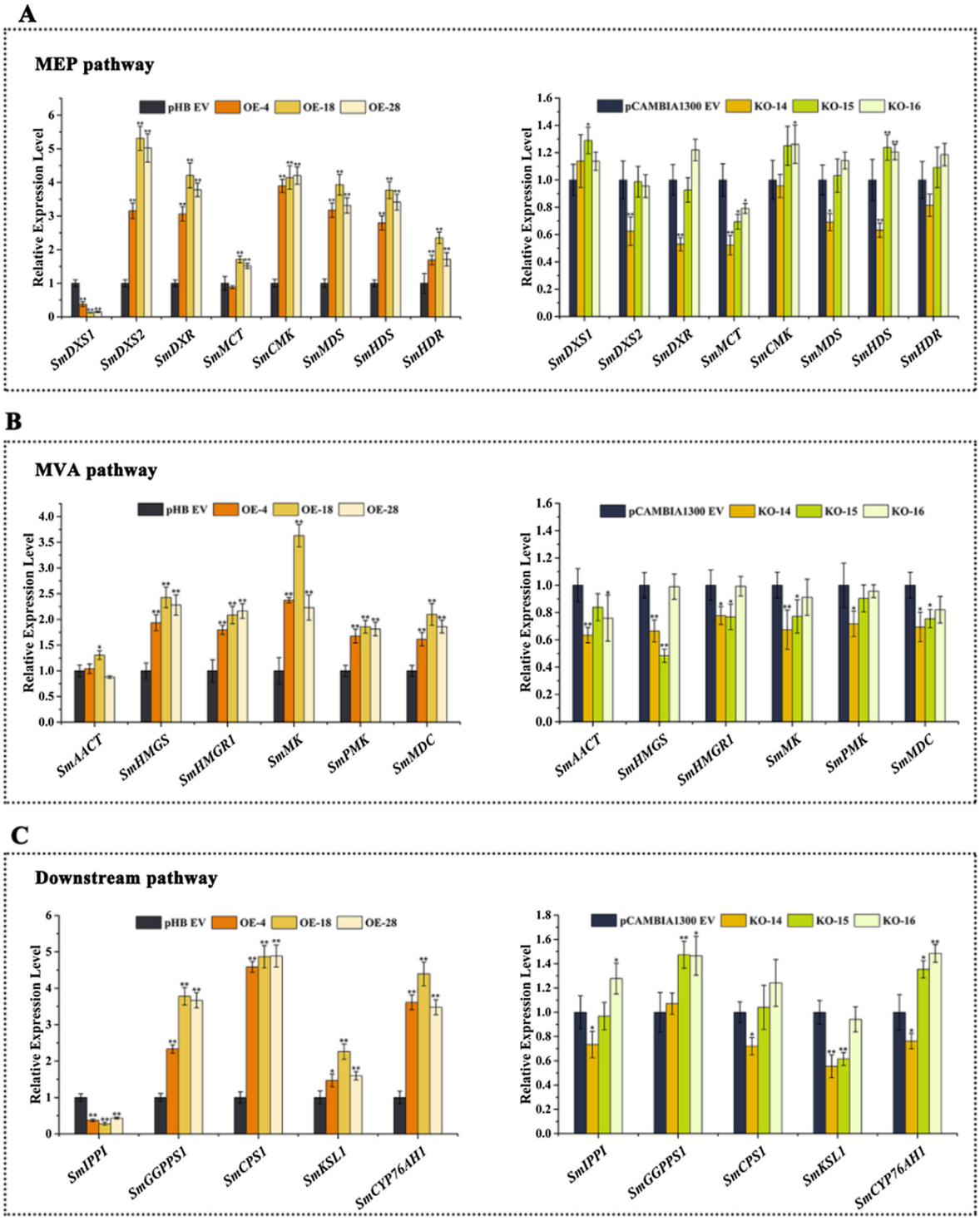

Fig. 7. Transcriptional expression analysis of tanshinone biosynthetic genes in selected *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines. Transcriptional expression levels of MEP pathway genes (A), MVA pathway genes (B), and downstream tanshinones biosynthetic genes (C) in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by qRT-PCR. The average transcriptional expression level of each gene in the two control hairy root lines was set to 1. The *S. miltiorrhiza actin* gene was used as the internal reference gene. Error bars represent the SD of three technical replicates.

shinone biosynthesis, the biosynthetic genes involved in the SA pathway have been partially illustrated [10]. The transcriptional expression levels of salvianolic acid biosynthetic genes in the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines were detected by qRT-PCR. Results revealed that the transcription levels of the four salvianolic acid biosynthesis pathway genes (i.e., *SmC4H1*, *SmPAL1*, *SmTAT1*, and *SmCYP98A14*) increased in all *SmMYB98-OE* transgenic hairy root lines and were >2.0-fold higher compared to the control (Fig. 8A). The expression levels of *SmCYP98A14* were slightly down-regulated in the *SmMYB98-KO* hairy root lines (Fig. 8B). These results further indicate that *SmMYB98* could be a positive regulator of salvianolic acid biosynthesis.

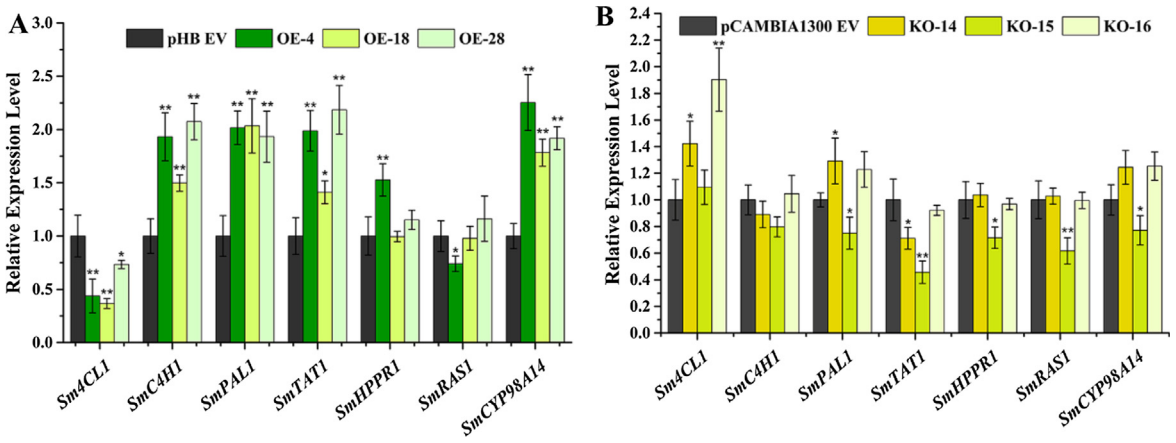

Fig. 8. Transcriptional expression analysis of salvianolic acid biosynthetic genes in selected *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines. (A, B) The transcriptional expression levels of salvianolic acid biosynthesis genes in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by qRT-PCR. The average transcriptional expression level of each gene in the two control hairy root lines was set to 1. The *S. miltiorrhiza actin* gene was used as the internal reference gene. Error bars represent the SD of three technical replicates.

SmMYB98 transactivates tanshinone and salvianolic acid biosynthetic genes

In order to investigate whether SmMYB98 could activate the expression of tanshinone and salvianolic acid biosynthetic genes, transient Dual-LUC assays were performed using tobacco leaves. *SmMYB98* was driven by the 35S promoter; the YFP construct was used as the negative control. Fourteen reporter constructs were produced by inserting the promoters of seven tanshinone biosynthetic genes (i.e., *SmDXS2*, *SmDXR*, *SmHMGR1*, *SmGGPPS1*, *SmCPS1*, *SmKSL1*, and *SmCYP76AH1*) and seven salvianolic acid biosynthetic genes (i.e., *SmPAL1*, *SmC4H1*, *Sm4CL1*, *SmTAT1*, *SmHPPR1*, *SmRAS1*, and *SmCYP98A14*) into the pGreenII 0800-LUC vector. Sets of effector and reporter constructs were co-infiltrated into tobacco leaves; the control groups were co-infiltrated into the opposite position of the same leaf. SmMYB98 activated the expression of *SmGGPPS1* in tanshinone biosynthesis, which was 2.7 fold higher in the ratio of LUC activities to REN activities (LUC/REN) compared to the YFP control, and activated *SmPAL1* and *SmRAS1* in salvianolic acid biosynthesis, which was 2.3- and 9.6-fold higher compared to the control, respectively (Fig. 9). Collectively, these results indicate that *SmMYB98* is a strong candidate gene with the ability to transactivate the *SmGGPPS1*, *SmPAL1*, and *SmRAS1* promoters.

Analysis of GA biosynthesis in the SmMYB98-OE and SmMYB98-KO hairy root lines

In order to verify whether *SmMYB98* could regulate GA biosynthesis, the GA content and expression levels of GA biosynthetic genes in the *SmMYB98-OE* and *SmMYB98-KO* hairy root lines were also detected. The GA content decreased in the OE lines and increased in the KO lines, which contrasted the tanshinone contents (Fig. 10A and C). The expressions of two GA biosynthesis genes (i.e., *SmKAO2* and *SmGA$_{20}$ox2*) decreased significantly in the *SmMYB98-OE* hairy root lines, which led to the synthesis of GA_3 compounds (Fig. 10B). The expression levels of *SmGA$_2$ox5* and *SmGA$_2$ox11* decomposed GA_3 biosynthesis and increased by more than two-fold in the OE lines compared to the control (Fig. 10B). *SmGA$_{20}$ox2* was slightly upregulated in the KO lines; there was no difference in the GA_3 decomposition genes (Fig. 10D). These results suggest that *SmMYB98* could be a negative regulator of GA biosynthesis by inhibiting GA synthesis and activating the expression of GA_3 decomposition genes.

As GAs could be involved in root growth, the *SmMYB98-OE* lines exhibited shorter and thicker hairy roots, while longer roots were observed in the *SmMYB98-KO* lines compared to the control (Fig. S9). After culturing in 1/2 MS liquid medium for two months,

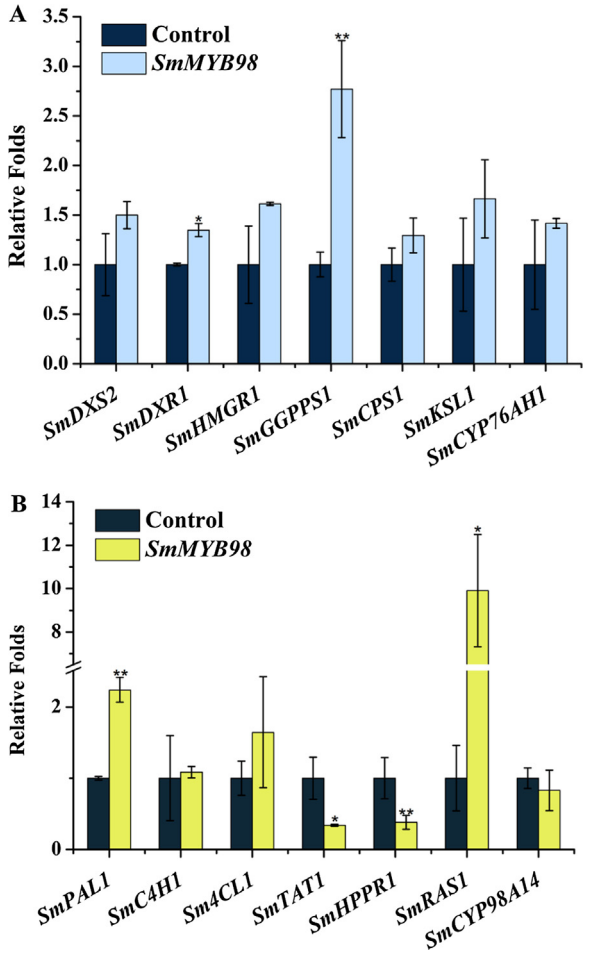

Fig. 9. The effects of SmMYB98 on the promoter of tanshinone (A) and salvianolic acid (B) biosynthetic genes were detected by transient Dual-LUC analysis using *N. benthamiana* leaves. The relative folds of LUC/REN represent the activation level of SmMYB98 on the promoters. Error bars represent the SD of three biological replicates.

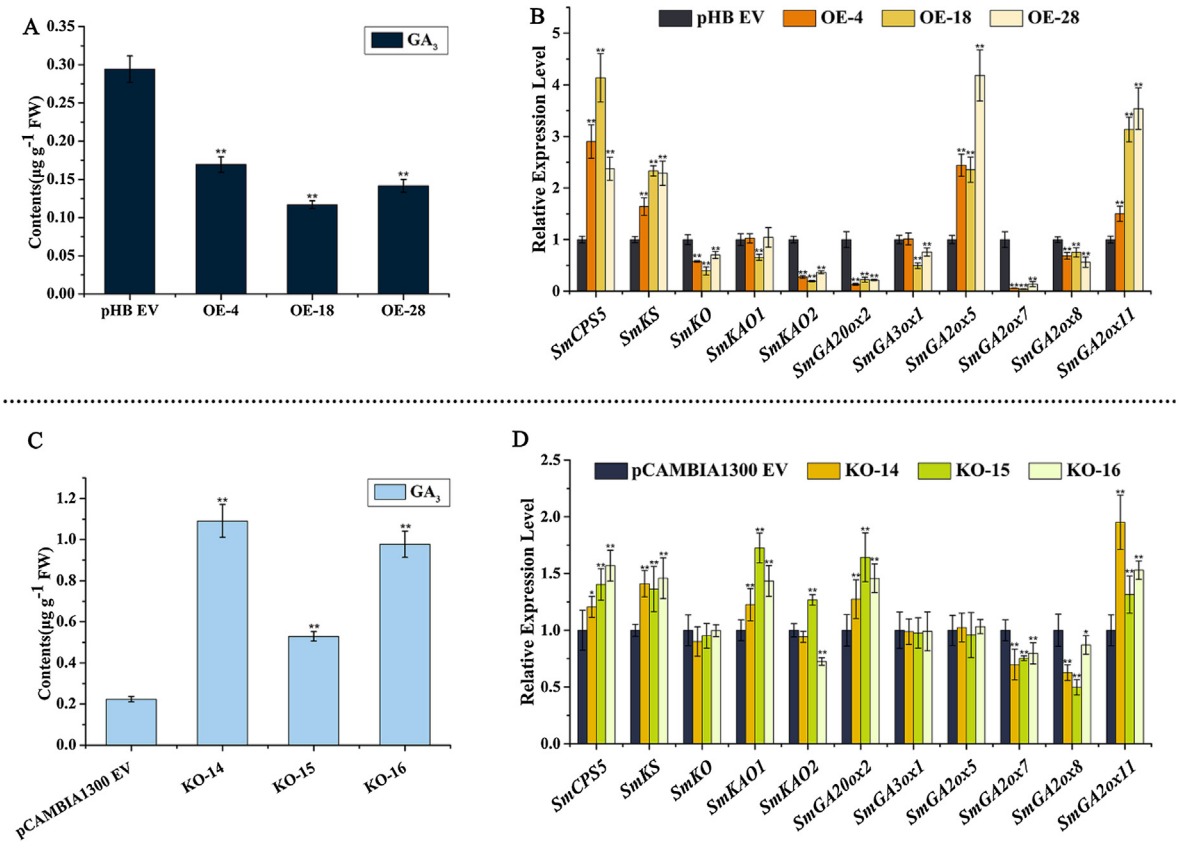

Fig. 10. Analysis of GA biosynthesis in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines. (A, C) The GA contents in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by HPLC. Error bars represent the SD of three technical replicates. (B, D) The transcriptional expression levels of GA biosynthetic genes in the *SmMYB98-OE* and *SmMYB98-KO* transgenic hairy root lines were detected by qRT-PCR. The average transcriptional expression level of each gene in the two control hairy root lines was set to 1. The *S. miltiorrhiza actin* gene was used as the internal reference gene. Error bars represent the SD of three technical replicates.

the *SmMYB98-OE* lines significantly increased the biomass of hairy roots (Fig. 6A, Table S2). In the *SmMYB98-KO* hairy root lines, the hairy root biomass was similar to the control with no significant increase or decrease (Fig. 6D, Table S2). Thus, GA biosynthesis was reduced in the *SmMYB98-OE* lines, which exhibited shorter and thicker roots, higher hairy root biomass, and higher tanshinone and salvianolic acid production (Fig. S9, Fig. 6A, Table S2). These results indicate that *SmMYB98* could alter the morphological structure of hairy roots, positively regulate tanshinone and salvianolic acid biosynthesis, and play a negative regulatory role in GA biosynthesis.

Discussion

MYB TF functions in tanshinone and salvianolic acid biosynthesis

The MYB family is one of the largest plant TF families in plants and is divided into four subfamilies, including 4R-MYBs, 3R-MYBs, R2R3-MYBs, and 1R-MYB [23]. Among them, the R2R3-MYB subfamily, which is comprised of the R2 and R3 MYB conserved domains, is the largest MYB subfamily. The functions of the R2R3-MYBs have been well-characterized, including plant developmental processes, responses to stress, and primary and secondary metabolism [22,24]. In *S. miltiorrhiza*, the 110 R2R3-MYB proteins have been identified and classified into 37 subgroups [25,26]. Members of the S3, S4, S5, S6, S7, S13, and S21 subgroups may regulate phenylpropanoid biosynthesis; four genes that belong to the S20 subgroup are presumed to participate in terpenoid biosynthesis. Specifically, *SmMYB39*, a S4 R2R3-MYB member, has been shown to inhibit phenolic acid biosynthesis [40]. An S5 R2R3-MYB member, *SmPAP1*, has been identified to promote phenolic acid and flavonoid biosynthesis [41]. *SmMYB9b* has been identified and verified to be an activator of tanshinone biosynthesis [27]. *SmMYB36* promotes tanshinone accumulation and reduces phenolic acid contents in transgenic *S. miltiorrhiza* hairy root lines [42]. Furthermore, *SmMYB111* regulates phenolic acid biosynthesis in *S. miltiorrhiza* by interacting with SmTTG1 and SmbHLH51 [43].

In this study, SmMYB98, an S20 R2R3-MYB TF, was subcelluarly localized in the nucleus and preferentially expressed in *S. miltiorrhiza* lateral roots. *SmMYB98* regulates tanshinone and salvianolic acid biosynthesis, which could promote tanshinone and salvianolic acid accumulation when *SmMYB98* is overexpressed. Furthermore, *SmMYB98* negatively regulated GA biosynthesis. Zhang et al. (2007) reported that *SmMYB9b-OE*, another S20 R2R3-MYB member, resulted in the significant improvement of tanshinones in *S. miltiorrhiza* hairy roots [27]. *SmMYB9b* was mainly expressed in flowers, while *SmMYB98* was mainly expressed in lateral roots where tanshinones accumulate [27]. Additionally, some candidate targets of SmMYB98 that regulate tanshinone and salvianolic acid biosynthesis through Dual-LUC assays were screened in this study to investigate the molecular regulation mechanism.

Relationship between the biosynthesis of different metabolites in S. miltiorrhiza

Tanshinones and salvianolic acids are two types of major bioactive compounds predominantly found in *S. miltiorrhiza*. It has been reported that some TFs are involved in the transcriptional

regulation of their biosynthesis. In these reported TFs, five R2R3-MYB TFs (i.e., *SmMYB39*, *SmPAP1*, *SmMYB36*, *SmMYB111*, and *SmMYB9b*) regulate the biosynthesis of tanshinones and salvianolic acids in *S. miltiorrhiza* [27,40–43]. Additionally, it was reported that *SmMYC2a* and *SmMYC2b*, two bHLH TFs, play similar roles in *S. miltiorrhiza* hairy root tanshinone and phenolic acid biosynthesis [29]. *SmWRKY1*, a WRKY TF, positively regulated tanshinone biosynthesis in *S. miltiorrhiza* hairy roots through *SmDXR* in the MEP pathway [44]. *SmERF1L1* and *SmERF115*, two ERF TFs, were also reported to regulate tanshinone and phenolic acid biosynthesis in *S. miltiorrhiza* [5,11].

GA, a type of phytohormone, plays an important role in many different aspects of plant growth and development [20,21,39,45,46]. Studies on the biosynthesis and regulation of GAs have helped demonstrate their role in the growth and development of *S. miltiorrhiza*, as well as elucidate the relationship between tanshinone and GA biosynthesis in *S. miltiorrhiza* [20,21]. Recently, the genes involved in the conversion of GGPP to GAs have been adequately studied in *S. miltiorrhiza* [20,21]. However, little is known about the regulatory mechanisms of GA biosynthesis in *S. miltiorrhiza*.

Similar to tanshinones, GAs are also biosynthesized from GGPP, a diphosphate precursor, and produced mainly in the MEP pathway of plastids and in the MVA pathway of cytosol. In this study, *SmMYB98* was involved in tanshinone biosynthesis, and revealed that the total tanshinone content in *SmMYB98-OE-18* increased to 15.4 mg/g DW, 3-fold higher compared to the control. The expression levels of the genes in the MEP pathway, MVA pathway, and *SmGGPPS1*, *SmCPS1*, *SmKSL1*, and *SmCYP76AH1* increased significantly. However, the expression levels of GA biosynthesis genes decreased significantly and the expression levels of GA deactivation genes increased when *SmMYB98* was overexpressed.

In terms of metabolic biosynthesis, GAs and tanshinones share the same diterpene biosynthesis pathway, so the reduction of GAs may theoretically improve the metabolic biosynthesis of tanshinones in *SmMYB98-OE* hairy root lines. However, the GA contents were very low in hairy roots, therefore this could not explain why tanshinones and salvianolic acids improved. In this study, the *SmMYB98-OE* lines reduced GA biosynthesis, exhibited shorter and thicker roots, and possessed a higher root biomass. *SmMYB98-OE* lines increased the biosynthesis of tanshinones and salvianolic acids, which was mainly achieved by promoting the expression of biosynthetic genes. Therefore, the reduction of GAs and increase of tanshinones and salvianolic acids were two results caused by *SmMYB98-OE* in *S. miltiorrhiza* hairy roots. The relationship between these two results has important theoretical significance and application value that could be further clarified and explained through further research.

Conclusion

In this study, a R2R3-MYB subgroup 20 TF, *SmMYB98*, simultaneously regulated the biosynthesis of tanshinones and salvianolic acids in *S. miltiorrhiza* hairy roots. *SmMYB98-OE* lines exhibited improved tanshinone and salvianolic acid contents, while reduced tanshinone and salvianolic acid contents were detected in the *SmMYB98-KO* hairy root lines. In accordance with the metabolism measurements, the transcriptional expression levels of tanshinone and salvianolic acid biosynthesis genes were upregulated in *SmMYB98-OE* lines and downregulated in *SmMYB98-KO* lines. Thus, it appears that *SmMYB98* may regulate tanshinone and salvianolic acid biosynthesis in *S. miltiorrhiza*. The present findings illustrate the exploitation of R2R3-MYB in terpenoid and phenolic biosynthesis, and provide a feasible strategy for improving tanshinone and salvianolic acid contents by MYB proteins in *S. miltiorrhiza*.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgments

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Declaration of Competing Interest

The authors have declared no conflict of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.01.012.

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