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 tanshinone and salvianolic acid biosynthetic genes.
- SmMYB98 negatively regulates the biosynthesis of gibberellins in *S. miltiorrhiza*.

**GRAPHICAL ABSTRACT**

**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...
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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.

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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 GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox), 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.
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, HPPP, 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 SmMYB89) were predicted to participate in terpenoid biosynthesis [25]. Consistent with this deduction, SmMYB89 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*.

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) *Raphanus sativus* (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_01563684.1) 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^ΔΔCt 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, pRI44, 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 (pSmDX2::fLUC, pSmDXR::fLUC, pSmHMGR1::fLUC, pSmGGPS1::fLUC, pSmCPS1::fLUC, pSmKSL1::fLUC, pSmCYP76AH1::fLUC, pSmPAL1::fLUC, pSmC4H1::fLUC, pSm4C1L::fLUC, pSmTAT1::fLUC, pSmHPPR1::fLUC, pSmRAS1::fLUC and pSmCYP98A14::fLUC) were obtained by inserting the promoters of seven tanshinone biosynthetic genes and seven salvinanolic 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 SmMYB62 (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* leaves 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 SmCY-P76AH1, 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 pH vector fused with the YFP reporter gene under the 35S promoter, to produce the pHBSmMYB98-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...
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. 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 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.
shinone biosynthesis, the biosynthetic genes involved in the SA pathway have been partially illustrated [10]. The 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.

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.

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.
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, SmCP51, 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 SmGA20ox2) decreased significantly in the SmMYB98-OE hairy root lines, which led to the synthesis of GA3 compounds (Fig. 10B). The expression levels of SmGA3ox5 and SmGA3ox11 decomposed GA3 biosynthesis and increased by more than two-fold in the OE lines compared to the control (Fig. 10B). SmGA3ox2 was slightly upregulated in the KO lines; there was no difference in the GA3 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 GA3 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,
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 SmHLH51 [43].

In this study, SmMYB98, an S20 R2R3-MYB TF, was subcellularly 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 SmMYB98) 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 hair 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 SmERF1L5, 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 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, SmCP5, SmSK1, 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 diterpen 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 explored 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.

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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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