Original Article

bHLH transcription factor SmbHLH92 negatively regulates biosynthesis of phenolic acids and tanshinones in Salvia miltiorrhiza

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Abstract

Objective: Salvia miltiorrhiza is a valuable herbal medicine with tanshinone and phenolic acid as the main biological active ingredients. The biosynthetic regulation of these bioactive compounds is controlled by a set of transcription factors (TFs). The basic helix-loop-helix (bHLH) transcription factor plays an important role in various physiological and biochemical processes in plants. However, research on bHLH TFs regulating phenolic acid or tanshinone biosynthesis in S. miltiorrhiza is limited.

Methods: qRT-PCR was used for gene expression analysis. The subcellular localization of SmbHLH92 was detected by SmbHLH92-GFP transient transformation into tobacco leaves, and its fluorescence was observed using a confocal laser scanning microscope. The transcriptional activity of SmbHLH92 was confirmed in the AH109 yeast strain. RNA interference hairy roots of SmbHLH92-RNAi transgenic lines were obtained through Agrobacterium-mediated genetic transformation. Ultra performance liquid chromatography (UPLC) was used to detect the changes of phenolic acids and tanshinones.

Results: SmbHLH92 is a bHLH transcription factor that is highly expressed in the root and phloem of S. miltiorrhiza. The subcellular localization and transcriptional activity of SmbHLH92 indicated that SmbHLH92 was located in the nucleus and may be a transcription factor. RNA interference (RNAi) of SmbHLH92 in hairy roots of S. miltiorrhiza significantly increased the accumulation of phenolic acid and tanshinone. Quantitative RT-PCR (RT-qPCR) analysis showed the transcription level of genes encoding the key enzymes involved in the phenolic acid and tanshinone biosynthetic pathways was increased in the hairy roots of the SmbHLH92-RNAi transgenic line, comparing with the control line.

Conclusion: These data indicate that SmbHLH92 is a negative regulator involved in the regulation of phenolic acid and tanshinone biosynthesis in S. miltiorrhiza.

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1. Introduction

Salvia miltiorrhiza Bunge is an important medicinal plant belonging to the Labiatae family. The dried rhizomes of S. miltiorrhiza, also called Danshen in Chinese, is a traditional Chinese medicine with a long medical history. Phenolic acid and diterpenoid tanshinone are the main secondary metabolites with biological activity in S. miltiorrhiza (Li, Xu & Liu, 2018). Hydrophilic phenolic acids include salvianolic acid B (Sal B), rosmarinic acid (RA), salvianolic acid A (Sal A) and lithospermic acid (LA) (Wang, Morris-Natschke & Lee, 2007), with antioxidant ability to scavenge free radicals (Ho & Hong, 2011; Zhao et al., 2008). In addition to phenolic acids, more than 40 tanshinones were isolated from S. miltiorrhiza (Zhang et al., 2012). Lipophilic tanshinones include diterpenoid tanshinones I (DT-I), tanshinone I (T-I), tanshinone IIa (T-IIa) and cryptotanshinone (CT) (Li, Song, Liu, Hu & Wang, 2009).

The biosynthesis and regulation of phenolic acid and tanshinone have been widely studied and elucidated. Some transcription factors (TFs) are involved in the regulation of phenolic and tanshinone biosynthetic pathways. The bHLH transcription factor contains a basic region for DNA binding at the N-terminus and an HLH domain at the C-terminus to form homodimers or
heterodimers (Goossens, Mertens & Goossens, 2017), which bind to the G-box in the promoter of their target genes (Ezer et al., 2017). bHLH TF plays an important role in regulating plant growth and development, secondary metabolism, stress resistance and signal transduction in plants (Dong et al., 2014; Gajewska, Janiak, Kwasniewski, Kedzierski & Szarejko, 2018; Liu et al., 2015; Xu et al., 2014). In particular, a group of bHLHs regulate the biosynthesis of flavonoids, alkaloids, and terpenoids in plants. In Arabidopsis thaliana, the bHLH proteins TT8, GL3 and EGL3, together with WD40 repeat-containing protein TTG1 and MYB proteins, activated anthocyanin biosynthesis (Gonzalez, Zhao, Leavitt & Lloyd, 2008). In Catharanthus roseus, the bHLH iridoid synthase 1 (BIS1) transcription factor controlled the level of gene transcription in the early stages of alkaloid biosynthesis. Overexpression of BIS1 in suspension culture cells led to increased accumulation of cyclopentyl ether terpenes and monoterpenoid indole alkaloids (Van Moerkercke et al., 2015). NbbHLH1 and NbbHLH2 positively regulated nicotine biosynthesis in Nicotiana benthamiana (Todd, Liu, Polvi, Parmett & Page, 2010). AtMYC2 increased sesquiterpene production by binding to the promoters of TPS21 and TPS11 (the sesquiterpene synthase genes) and activating transcription of these two genes in A. thaliana (Hong, Xue, Mao, Wang & Chen, 2012).

In S. miltiorrhiza, several bHLH TFs have been identified to be involved in the regulation of bioactive compound biosynthesis. SmMYC2a and SmMYC2b, two TFs that interacted with JAZ, regulated the biosynthesis of tanshinone and Sal B in S. miltiorrhiza (Zhou et al., 2016). Overexpression of SmbHLH148 up-regulated the production of phenolic acids and tanshinones in hairy roots of S. miltiorrhiza (Xing et al., 2018a). SmbHLH10 up-regulated tanshinone biosynthesis by binding to the promoter and activating the expression levels of DXS2, CPS1 and CPS5 (Xing et al., 2018b). Ectopic expression of AtDAP1 led to high accumulation of Sal B in transgenic plants of S. miltiorrhiza (Zhang, Yan & Wang, 2010). SmbHLH51 was significantly up-regulated and positively regulated the biosynthesis of phenolic acids (Wu et al., 2018).

A total of 127 bHLH transcription factor genes have been identified in the genome of S. miltiorrhiza (Zhang et al., 2015). Seven bHLHs were expected to participate in the regulation of tanshinone biosynthesis (Zhang et al., 2015). Among these candidate bHLHs, the expression profile of SmbHLH92 in different organs and tissues exactly matched the accumulation pattern of tanshinone in S. miltiorrhiza (Zhang et al., 2015), suggesting that this gene may be related to the biosynthesis of active ingredients. To further characterize the molecular function of SmbHLH92, we cloned and identified SmbHLH92 from S. miltiorrhiza among these candidate bHLHs. Compared to the control line, RNAi-mediated SmbHLH92 silencing transgenic lines accumulated higher levels of phenolic acids (Sal B, Sal A, RA, and LA) and tanshinones (DT-I, CT, T-I, and T-II A) in the hairy roots of S. miltiorrhiza. Several key enzyme genes of phenolic acid and tanshinone biosynthetic pathways in hairy roots of SmbHLH92-RNAi were up-regulated. These data indicate that SmbHLH92 is a negative regulator of phenolic acid and tanshinone biosynthesis in S. miltiorrhiza.

2. Materials and methods

2.1. Plant materials and treatment

Salvia miltiorrhiza (line 99–3) was planted in the garden of Beijing Institute of Medicinal Plant Development (IMPLAD). The roots, stems, leaves and flowers of 2-year-old S. miltiorrhiza were collected, immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction. The roots were divided into three tissues, including the periderm, phloem, and xylem, for expression profiling analysis. N. benthamiana was grown in pots at (23 ± 2) °C under 16 h light / 8 h dark photoperiod, and watered every two days.

2.2. Cloning and phylogenetic analysis of SmbHLH92

A bHLH transcription factor named SmbHLH92 was amplified using the cDNA reverse transcribed from total RNA isolated from S. miltiorrhiza seedlings as previous study (Zhang et al., 2015). ExPASy and SMART were used to predict the molecular weight, isoelectric point and domain of SmbHLH92. The amino acid sequence of SmbHLH92 used as query sequences searched for homologs in NCBI (http://www.ncbi.nlm.nih.gov/) by BLASTP, and the evolutionary relationship between SmbHLH92 and homologues was established. Multiple alignments were generated on the DNAMAN software. A neighbor joining (NJ) tree with 1000 bootstrap repeats was constructed using MEGA 6.0 with the full-length amino acid sequences (Kumar, Tamura, Jakobsen & Nei, 2001; Saitou & Nei, 1987). Table S1 listed bHLH of other species.

2.3. Subcellular localization analysis

The full-length coding region of SmbHLH92 was fused with the green fluorescent protein (GFP) in the PCAMBIA1302-GFP vector, which was identified by PCR using mgFP and SmbHLH92-GFP specific primers. The expression vector was transiently transferred to Agrobacterium strain GV3101, which was injected into 5-week-old tobacco leaves. After 48 h of incubation, the confocal laser scanning microscope (Zeiss, LSM700) was used to observe the GFP fluorescence of SmbHLH92. The PCAMBIA1302 plasmid was transformed into tobacco leaves as a positive control. Nuclei were stained with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI). Table S2 listed the primers used for subcellular localization analysis.

2.4. Transcriptional activation activity analysis of SmbHLH92

The PCR product of SmbHLH92 was inserted into the Ndel and Smal restriction sites of the pgGBK7 vector to generate pGBK7T3-SmbHLH92. Empty pGBK7 was used as a negative control. According to the manufacturer’s protocol (Clontech, United States), pGBK7T3-SmbHLH92 and empty pGBK7 vectors were transformed into the Saccharomyces cerevisiae AH109. Subsequent transformants were verified by yeast colony PCR, and then inoculated onto synthetic dropout (SD) media lacking tryptophan (SD-Trp), and selected on SD medium without tryptophan, histidine and adenine (SD/-Trp/-His/-Ade) (diluted to 10–1, 10–2 yeast strain concentrations, respectively). The plate was then incubated at 30 °C for 3 d. Transcriptional activation activity was evaluated based on the growth status of yeast clones. Table S2 listed the primers used to analyze transcription activation activity.

2.5. Generation of transgenic SmbHLH92-RNAi hairy roots

The 123 bp sequence-specific fragment of SmbHLH92 was cloned from S. miltiorrhiza, corresponding to the SmbHLH92 cDNA from 529 bp to 651 bp. The PCR product was recombined into pDONR entry vector by using the BP reaction, and the PCR product was introduced into the expression vector pK7GW1WG2D (II) by using the LR reaction according to the protocol of Gateway (Invitrogen, United States). The recombinant plasmid was transformed into Agrobacterium rhizogenes (ACC10060) carrying Ri (root inducing) plasmid. Leaves of S. miltiorrhiza infected with ACC10060 produced hairy roots of transgenic lines. Positive transgenic lines were identified using PCR to detect products produced by amplification of rolC-, eGFP-, and SmbHLH92-specific primers. The control
line was a plant transformed with the corresponding empty vector (pkl) and identified by PCR to detect the genes of rolC and eGFP. Subcultures of hairy roots were then maintained in 6, 7-V liquid medium. The liquid culture medium containing hairy roots was placed on an orbital shaker at a speed of 120 rpm in the dark at 25 °C. Then, hairy roots were collected after 30 d for gene expression analysis using real-time quantitative PCR (RT-qPCR) method, and the content of phenolic acid and tanshinone was analyzed by UPLC after culturing for five months. Table S2 listed the primers used for vector construction and hairy root identification.

2.6. Gene expression analysis with RT-qPCR

The expression level of SmbHLH92 in different organs/tissues and in SmbHLH92-RNAi transgenic hairy roots was analyzed by RT-qPCR. Total RNA was extracted from different organs and tissues in S. miltiorrhiza according to the manufacturer’s instructions of RNasep Pure Plant Kit (TIANGEN, China), and converted into cDNA by using PrimeScript® 2.0 First-Strand cDNA Synthesis Kit (Takara, Japan). Organs and tissues included the roots, stems, leaves, flowers, periderm, phloem, and xylem of a 2-year-old S. miltiorrhiza. RT-qPCR amplification was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosys) using SYBR Premix Ex TaqTM (Takara, Beijing, China). The RT-qPCR cycle program includes: 95 °C for 30 s, 1 cycle; 95 °C for 5 s; and 60 °C for 34 s, 40 cycles. Relative gene expression was calculated using the 2−ΔΔCT method (Livak & Schmittgen, 2001). The SmACTIN gene (HM231319.1) was used as an internal reference. The experiments were performed in three independent biological experiments with three technical replicates.

RT-qPCR was performed to determine the expression levels of several key enzyme genes of the phenolic acid biosynthetic pathway, such as TATI, PALI, C4H1, 4CL2, HPRP1, RAS1 and CYP76A14. In addition, key enzyme genes of the tanshinone biosynthetic pathway, such as CPS1, KSI1, DKS2, CYP76A8H1, CYP76A8H3 and CYP76A8K1, were also evaluated. The primers used in RT-qPCR were listed in Table S3.

2.7. Metabolite analysis by UPLC

The 5-month-old hairy roots were collected and dried to constant weight in an oven at 40 °C. The sample powder was divided into two parts. The first part was extracted with 2 mL of 75% methanol with 100 mg of powder for phenolic acid test; the second part was extracted with 5 mL of methanol with 1000 mg of powder for tanshinone test under ultrasonic treatment for 30 min. Then, the powder was centrifuged at 8000 × g for 10 min, and then filtered through a 0.22 μm microporous membrane filter. The Waters UPLC system (Waters, USA) equipped with a PDA detector was used to determine the content of phenolic acid and tanshinone. An ACQUITY BEH C18 column (2.1 mm × 100 mm, 1.7 μm; Waters) was used. Phenolic and tanshinone were detected using 280 and 255 nm PDA wavelengths, respectively. Phenolic acid was detected by selective gradient elution using mobile phase acetonitrile (A)–0.5% formic acid (B) (volume percent, in deionized water). Linear gradients included 5%–25% A (0–10 min), 25%–40% A (10–20 min), 40%–90% A (20–25 min), and 90% A (25–30 min). The flow rate was 0.3 mL/min. Gradient elution using mobile phase methanol (A)–water (B) was used to detect tanshinone. The linear gradient included 20%–60% A (0–5 min), 60%–70% A (5–20 min), 70%–80% A (20–25 min), 80%–100% A (25–26 min), and 100% A (26–30 min). The flow rate was 0.25 mL/min.

3. Results

3.1. Cloning and phylogenetic analysis of SmbHLH92

Based on the sequence of SmbHLH92 in S. miltiorrhiza genome, the full-length sequence of SmbHLH92 was obtained (GenBank accession number: KP257525.1). SmbHLH92 was 666 bp in length and encoded 221 amino acids, with a calculated molecular weight of 24.96 kDa and an isoelectric point of 6.6. Multiple alignments of SmbHLH92 amino acids and SMART analysis revealed the conserved BHLH domain (58–110 amino acids) present at the N-terminus of SmHLH92 (Fig. 1A). SmbHLH92, along with the bHLH proteins from S. miltiorrhiza, A. thaliana, Nicotiana tabacum, N. ben- thamiana, C. roseus, Cucumis sativus, Taxus cuspidate, Coptis japonica, Vitis vinifera, Petunia hybrida, Ipomoea purpurea and Pyrus pyri- folia were used to construct a phylogenetic tree. SmbHLH92 and Bt proteins were highly homologous (Fig. 1B). Bt (bitter leaf) and Bt (bitter fruit) transcription factors can regulate the concentration of cucurbitacin C in cucumber (Shang et al., 2014). Since the cucurbitacin is triterpenoids conferring a bitter taste in cucurbit, suggesting that SmbHLH92 may play a role in regulating terpene biosynthesis in S. miltiorrhiza.
Fig. 2. Expression analysis of SmbHLH92 in S. miltiorrhiza. (A) Expression pattern of SmbHLH92 in different organs/tissues of S. miltiorrhiza. These organs/tissues include root (R), stem (S), leaf (L), flower (F), periderm (R1), phloem (R2), and xylem (R3). (B) Subcellular localization of SmbHLH92 protein in tobacco (N. benthamiana). The fluorescence was observed using a confocal laser scanning microscope. The picture shows GFP, DAPI, bright field (bright) and superposition (merging) of three fields. GFP and 92-GFP bars were 50 and 30 μm, respectively.

3.2. Expression patterns of SmbHLH92

Gene expression patterns are often related to gene function. SmbHLH92 was mainly expressed in the root and phloem, while its transcript was hardly detected in the stem (Fig. 2A). SmbHLH92 was observed to be expressed in large amounts in the roots of S. miltiorrhiza, and the root is the main part of the accumulation of active ingredients in S. miltiorrhiza (Zhang et al., 2015). This root-specific high expression pattern suggests that SmbHLH92 may be involved in regulating the biosynthesis of active ingredients in S. miltiorrhiza.

3.3. Subcellular localization and transcriptional activation activity analysis of SmbHLH92

The fusion protein pCAMBIA1302-SmbHLH92-GFP was constructed and transiently expressed in N. benthamiana to reveal the subcellular localization of SmbHLH92 in cells. In contrast to the GFP control protein, which showed widespread green fluorescence throughout the cell, the SmbHLH92-GFP fusion protein was localized in the nucleus of tobacco leaf cells. DAPI staining confirmed this finding (Fig. 2B), suggesting that SmbHLH92 was a nuclear localized protein.

We constructed a recombinant vector pGBK7-SmbHLH92 and analyzed the transcriptional activity of SmbHLH92 in AH109 yeast strain (Fig. 3A). The results showed that the yeast containing pGBK7-SmbHLH92 was able to survive on SD/Trp and SD/Trp-His-Ade media (Fig. 3B), indicating that SmbHLH92 had transcriptional activity.

3.4. Generation of SmbHLH92 transgenic hairy roots

To investigate the biological function of SmbHLH92 in S. miltiorrhiza, we constructed RNAi transgenic hairy root lines using the Gateway system. Three independent RNAi transgenic hairy root lines were selected in kanamycin-containing medium, and gene expression levels of SmbHLH92 in these RNAi and control lines were confirmed by RT-qPCR. Compared with the control transgenic...
Fig. 4. Expression of SmbHLH92 in *S. miltiorrhiza* hairy root transgenic lines. (A) Relative quantitative analysis of SmbHLH92 expression in transgenic lines and control of *S. miltiorrhiza* hairy roots. The results were analyzed using the 2^−ΔΔCT_ method. SmACTIN is used as an internal reference. Bars are represented as means ± SD from three independent biological replicates. Statistical significance was performed using Student’s t-test (n.s.: P > 0.05; *: 0.01 < P < 0.05; **: P < 0.01). (B) Hairy roots of *S. miltiorrhiza* induced by *Agrobacterium rhizogenes* (ACCC10060). Hairy roots were cultured in 6, 7-V liquid medium for 5 months before being photographed.

Fig. 5. Content of phenolic acid and tanshinone in hairy roots of SmbHLH92-RNAi. (A)–(D) Contents of RA (A), LA (B), Sal B (C), and Sal A (D) in the transgenic and control hairy roots of *S. miltiorrhiza*. (E)–(H) Contents of DT-I (E), CT (F), T-I (G), and T-IIA (H) in hairy roots of transgenic and control lines of *S. miltiorrhiza*. Bars are represented as means ± SD from three independent biological replicates. Statistical significance was evaluated with Student’s t-test (n.s.: P > 0.05; *: 0.01 < P < 0.05; **: P < 0.01).
line (pki), the transcription levels of SmbHLH92 in the three RNAi lines, namely 92i-4, 92i-5, and 92i-10, were reduced by 3.30, 1.82, and 1.85 times, respectively (Fig. 4A), indicating that the construction of the SmbHLH92-RNAi transgenic line was successful. After 5 months of liquid culture, the phenotypes of these RNAi transgenic lines and control line were the same (Fig. 4B), indicating that RNAi of SmbHLH92 had no effect on the growth and development of S. miltiorrhiza.

3.5. RNAi of SmbHLH92 increases the concentration of phenolic acids and tanshinones in hairy roots of S. miltiorrhiza

Given that the root-specific expression profile of SmbHLH92 was consistent with the accumulation pattern of phenolic acid and tanshinone in S. miltiorrhiza, we predicted that this gene may play a regulatory role in the biosynthesis of these active ingredients in S. miltiorrhiza. The content of several typical compounds of phenolic acids (Sal B, Sal A, RA, and LA) and tanshinone (DT-1, CT, T-1, and T-IIA) were evaluated in control and SmbHLH92-RNAi transgenic lines. Compared with the control line, the phenolic acid concentrations in the three SmbHLH92-RNAi lines were all significantly increased (P < 0.05) (Figs. 5A-D). For example, the content of RA, Sal B, Sal A, and LA in the 92i-5 line reached 1.85+, 1.52+, 1.55+, and 1.46+ times of the control line, respectively (Figs. 5A-D). Tanshinone content was also increased in these SmbHLH92-RNAi lines. For example, the CT content in the 92i-4, 92i-5, and 92i-10 lines was 2.60+, 2.31+, and 2.65+ times higher than that of the control line, respectively. The concentrations of DT-1 were 2.01+, 2.06+, and 2.23+ times higher than those in the control line, respectively (Figs. 5E-H).

These results indicated that SmbHLH92 plays an important role in the regulation of phenolic and tanshinone biosynthetic pathways and acts as an inhibitor that inhibits the accumulation of these bioactive compounds in S. miltiorrhiza. Table S4 summarized all the contents of phenolic acid and tanshinone compounds. Typical UPLC profiles of phenolic acid (Fig. 6A) and tanshinone (Fig. 6B) in control and SmbHLH92-RNAi transgenic hairy roots clearly showed the differences in the content of these compounds in these transgenic lines.

3.6. SmbHLH92 RNAi affects gene expression in phenolic acid and tanshinone biosynthetic pathways

To reveal the potential regulatory mechanisms of SmbHLH92 on phenolic acid and tanshinone accumulation, a set of genes encoding key enzymes involved in the biosynthetic pathway of these compounds were selected to determine the relative expression levels in the control and SmbHLH92-RNAi transgenic lines. Relative expression levels of TAT1 (DQ334606.1), HPPR1 (DQ099741.1), PAL1 (EF462460.1), C4H1 (DQ355979.1), 4CL2 (AY237164.1), RAS1 (FJ906696.1), and CYP98A14 (HQ316179.1), which are involved in the phenolic acid biosynthetic pathway, were examined to illustrate the regulatory mechanism of SmbHLH92 on the biosynthesis of phenolic acids. The relative expression levels of CYP98A14 reached 3.02+ (92i-5) and 3.54+ times (92i-10), while PAL1 reached 2.67+ (92i-5), and 2.60+ times (92i-10) in the SmbHLH92-RNAi transgenic lines compared to the control (Fig. 7).

Key enzyme-encoding genes in the tanshinone biosynthetic pathway include DXS2 (FJ643618.1) in the MEP pathway, CPS1 (EU003997.1), KSL1 (EF635966.2), CYP76AH1 (JX422213.1), CYP76AH3 (KR140168.1), and CYP76AK1 (KR140169.1) in the downstream pathway were also evaluated. These genes were up-regulated in SmbHLH92-RNAi lines compared to the control line, excepted the line of 92i-4 (Fig. 8). For example, compared to the expression level in the control line, the abundance of CYP76AH3 transcripts reached 5.24+ (92i-5), and 7.56+ (92i-10) times (Fig. 8). The expression changes of these genes in the phenolic acid and tanshinone biosynthetic pathways are consistent with the accumulation of these active ingredients in S. miltiorrhiza. These results indicated that SmbHLH92 may hinder the expression of key genes, thereby inhibiting the biosynthesis of phenolic acid and tanshinone in S. miltiorrhiza.

4. Discussion

Transcription factors regulate the biosynthesis of secondary metabolites by regulating the expression of key enzyme genes in

Fig. 6. Typical UPLC curves for phenolic acid and tanshinone. (A) Typical UPLC spectrum of phenolic acid in hairy roots of SmbHLH92-RNAi. The four main compounds are labeled 1–4, identified as RA, LA, Sal B, and Sal A. (B) Typical UPLC spectrum of tanshinone in hairy roots of SmbHLH92-RNAi. The four main compounds are labeled 5–8, identified as DT-1, CT, T-1, and T-IIA.
Fig. 7. Relative expression levels of phenolic acid biosynthetic pathway genes in hairy root transgenes and control lines. All values are expressed as mean ± SD. Statistical significance is assessed by Student's t-test (n.s.: $P > 0.05$; *: $0.01 < P < 0.05$; **: $P < 0.01$).

Fig. 8. Relative expression levels of tanshinone biosynthetic pathway genes in hairy root transgenes and control lines. All values are expressed as means ± SD. Statistical significance is assessed with Student's t-test (n.s.: $P > 0.05$; *: $0.01 < P < 0.05$; **: $P < 0.01$).
the biosynthetic pathway in medicinal plants. Many transcription factors found in the herbal genome play important roles in regulating the biosynthesis of bioactive compounds (Xin et al., 2019). The bHLH transcription factor binds to the G-box in the promoter of the target gene, thereby activating or inhibiting the transcription of key genes to regulate the synthesis of active ingredients. A total of 127 bHLH genes in S. miltiorrhiza have been identified by genome-wide analysis (Zhang et al., 2015). Among these genes, seven bHLHs were predicted to be involved in tanshinone biosynthesis, including SmbHLH92 and SmbHLH37. Recently, SmbHLH37 was identified as a new target for JAZ protein and a negative regulator of Sal B biosynthesis, which antagonized SmMYC2 by binding to the promoters of the target genes of SmTAT1 and SmPAL (Du et al., 2018). In this study, RNAi-mediated SmbHLH92 silenced transgenic lines increased accumulation of phenolic acids and tanshinones in hairy roots of S. miltiorrhiza. Therefore, SmbHLH92 was proved to be the first transcription factor that negatively regulates tanshinone and phenolic acid synthesis in S. miltiorrhiza.

4.1. SmbHLH92 acts as a putative transcription factor

The N-terminus of SmbHLH92 contains a conserved domain that defines the bHLH transcription factor (Fig. 1A), indicating that this gene belongs to the bHLH gene family. In addition, the green fluorescence of the SmbHLH92-GFP fusion protein was concentrated in the nucleus of the tobacco leaf cells. DAPI staining also confirmed the fluorescent in nuclei (Fig. 2B). The nuclear localization of SmbHLH92 is consistent with the characteristics of transcription factors. SmbHLH92 has shown transcriptional activity in the yeast system, which supports its putative function as a transcription factor. In addition, phylogenetic analysis revealed that SmbHLH92 is highly homologous to Bt protein and BIS1 (Fig. 1B), suggesting that SmbHLH92 may be a transcription factor that regulates terpene biosynthesis in S. miltiorrhiza.

4.2. SmbHLH92 inhibits the expression of enzyme-encoding genes to regulate the biosynthesis of phenolic acid and tanshinone in S. miltiorrhiza

Transcription factors co-expressed with key enzyme genes responsible for the biosynthesis of bioactive secondary metabolites may play an important role in regulating the biosynthesis of these compounds. The active ingredients of S. miltiorrhiza accumulate mainly in the roots (Xu et al., 2015). SmbHLH92 transcripts are abundant in the root and phloem (Fig. 2A), suggesting that SmbHLH92 is an important regulator of phenolic acid and tanshinone biosynthesis. The content of phenolic acids (Sal B, Sal A, RA and LA) and tanshinones (DT-I, CT, T-I and T-IIa) in SmbHLH92-RNAi hairy roots increased significantly (Fig. 5), indicating that SmbHLH92 acts as a repressor involved in regulating the accumulation of phenolic acids and tanshinones in S. miltiorrhiza. Unlike previously identified SmbHLH37, which only negatively regulates Sal B biosynthesis, SmbHLH92 negatively regulates both phenolic acid and tanshinone biosynthesis.

Phenolic acids are synthesized via the phenylpropanoid- and tyrosine-derived pathways. At least 29 genes are involved in the biosynthesis of phenolic acids in the S. miltiorrhiza genome (Wang et al., 2015). SmbHLH92 negatively regulated PAL1 in the phenylpropanoid pathway, negatively regulated TAT1 in the tyrosine-derived pathway, and negatively regulated RAS1 and CYP98A14 in the phenolic acid pathway (Fig. 7). These highly expressed genes encoding key enzymes responsible for phenolic acid biosynthesis contribute to the accumulation of phenolic acids in the hairy roots of SmbHLH92-RNAi lines.

Tanshinones are derived from the MEP and MVA pathways. The expression levels of key enzyme genes involved in tanshinone biosynthesis were detected in our study. In the SmbHLH92-RNAi lines, most key enzyme genes, such as DXS2 in the MEP pathway and CPS1, KSL1, CYP76A1H1 and CYP76A3H3 genes involved in the downstream pathway were all up-regulated (Fig. 8). However,
the expression level of genes related to tanshinone biosynthesis were lower in 92i-4 than that in the control line, which probably due to the instability of transgenic hairy roots. The results show that SmbHLH92 inhibits the biosynthesis of these compounds by inhibiting the expression of key enzyme genes, thereby regulating the biosynthesis of phenolic acid and tanshinone.

4.3. SmbHLH92 may indirectly regulate the accumulation of bioactive compounds in S. miltiorrhiza

Transcription factors interact with the cis-elements of key genes to regulate the biosynthetic pathways of secondary metabolites. The bHLH transcription factor activates or inhibits gene transcription by binding to the G-box element in the promoter of the target gene. We analyzed the G-box in the promoter (~ 1500 bp) region of genes involved in the biosynthetic pathways of phenolic and tanshinone biosynthetic pathways (including 4CL2, RAS1, TAT1, HPPR1, CPS1 and DXS2) (Table S5). In order to detect the target gene of SmbHLH92, we studied the direct binding activity of the transcription factor and the G-box motif in the promoters of two key enzyme-encoding genes TAT1 and RAS1, which are involved in the biosynthetic pathway of phenolic acids, using yeast One-Hybrid (YIH) method. However, YIH results indicate that SmbHLH92 does not directly bind to the promoters of TAT1 and RAS1 directly (Data unpublished). The target genes of SmMYC2a/SmMYC2b, SmbHLH10 and SmbHLH17 were CYP98A1a (Zhou et al., 2016), CPS1, CPS5 and DXS2 (Xing et al., 2018b), and TAT1 and PALI (Du et al., 2018) (Fig. 9). SmbHLH92 might inhibit the expression of CPS1 and 4CL2, which are probably the target genes of SmbHLH92, resulting to the negatively regulation the biosynthesis of tanshinone and phenolic acid in S. miltiorrhiza (Fig. 9). Identification of the target genes for SmbHLH92 is still in progress.

Since most genes in the phenolic acid and tanshinone biosynthetic pathways are induced in SmbHLH92-RNAi transgenic lines, we speculate that SmbHLH92 may regulate enzyme genes upstream of these pathways or interact indirectly with other proteins to control the synthesis of active substances in S. miltiorrhiza. bHLH transcription factors often interact with MYB family proteins to form complexes and mediate the regulation of target gene transcription (Feller, Machemer, Braun & Grotewold, 2011). The mechanism of SmbHLH92 participates in the regulation of biosynthesis of bioactive compounds needs further study.

Our research provides a reference to increase the production of phenolic acids and tanshinones in S. miltiorrhiza by using biological methods such as genetic engineering in the biosynthetic pathway (Shi et al., 2016). Further experiments should be performed to elucidate the precise molecular mechanism of SmbHLH92 in regulating phenolic and tanshinone biosynthesis in S. miltiorrhiza.

5. Conclusion

In this study, we cloned and characterized a new transcription factor, SmbHLH92, which has transcriptional activity with nuclear localization. Its transcript is the highest in the root and phloem of S. miltiorrhiza. RNA interference of SmbHLH92 significantly increased accumulation of phenolic acids and tanshinones in hairy root transgenic lines. The expression levels of several key enzyme genes of phenolic acid and tanshinone biosynthetic pathways in hairy root of SmbHLH92-RNAi were up-regulated. These data indicate that SmbHLH92 is a negative regulator of phenolic acid and tanshinone biosynthesis in S. miltiorrhiza. Overall, this study provides new insights into the role of bHLH in the regulation of biosynthesis of bioactive secondary metabolites in S. miltiorrhiza.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.chemmed.2020.04.001.

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