Chinese Herbal Medicines 12 (2020) 237–246

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

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

Jian-hong Zhang[a], Hai-zhou Lv[a], Wan-jing Liu[a], Ai-jia Ji[a,b], Xin Zhang[a], Jing-yuan Song[a], Hong-mei Luo[a,*], Shi-lin Chen[c]

[a] *Engineering Research Center of Chinese Medicine Resource, Ministry of Education, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China*
[b] *School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China*
[c] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*

ARTICLE INFO

Article history:
Received 16 August 2019
Revised 25 October 2019
Accepted 10 December 2019
Available online 19 April 2020

Keywords:
phenolic acid
Salvia miltiorrhiza bunge
SmbHLH92
tanshinone
transcription factor

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*.

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

* Corresponding author.
E-mail address: hmluo@implad.ac.cn (H.-m. Luo).

https://doi.org/10.1016/j.chmed.2020.04.001

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, Kedziorski & 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 synthesis 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 monoterpene indole alkaloids (Van Moerkercke et al., 2015). *NbbHLH1* and *NbbHLH2* positively regulated nicotine biosynthesis in *Nicotiana benthamiana* (Todd, Liu, Polvi, Pammett & 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 *AtPAP1* 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-IIA) 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* seedling 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 *NdeI* and *SmaI* restriction sites of the pGBKT7 vector to generate pGBKT7-*SmbHLH92*. Empty pGBKT7 was used as a negative control. According to the manufacturer's protocol (Clontech, United States), pGBKT7-*SmbHLH92* and empty pGBKT7 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°, 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 pK7GWIWG2D (II) by using the LR reaction according to the protocol of Gateway (Invitrogen, United States). The recombinant plasmid was transformed into *Agrobacterium rhizogenes* (*ACCC10060*) carrying Ri (root inducing) plasmid. Leaves of *S. miltiorrhiza* infected with *ACCC10060* 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 (pki) 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 and hairy roots of *S. miltiorrhiza* according to the manufacturer's instructions of RNAprep Pure Plant Kit (TIANGEN, China), and converted into cDNA by using PrimeScript® II 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 $2^{-\Delta\Delta CT}$ 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 *TAT1, PAL1, C4H1, 4CL2, HPPR1, RAS1* and *CYP98A14*. In addition, key enzyme genes of the tanshinone biosynthetic pathway, such as *CPS1, KSL1, DXS2, CYP76AH1, CYP76AH3* and *CYP76AK1*, 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 C_{18} 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 SmbHLH92 (Fig. 1A). SmbHLH92, along with the bHLH proteins from *S. miltiorrhiza, A. thaliana, Nicotiana tabacum, N. benthamiana, C. roseus, Cucumis sativus, Taxus cuspidate, Coptis japonica, Vitis vinfera, Petunia hybrida, Ipomoea purpurea* and *Pyrus pyrifolia* were used to construct a phylogenetic tree. SmbHLH92 and Bt proteins were highly homologous (Fig. 1B). Bl (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 cucurbits, suggesting that SmbHLH92 may play a role in regulating terpene biosynthesis in *S. miltiorrhiza*.

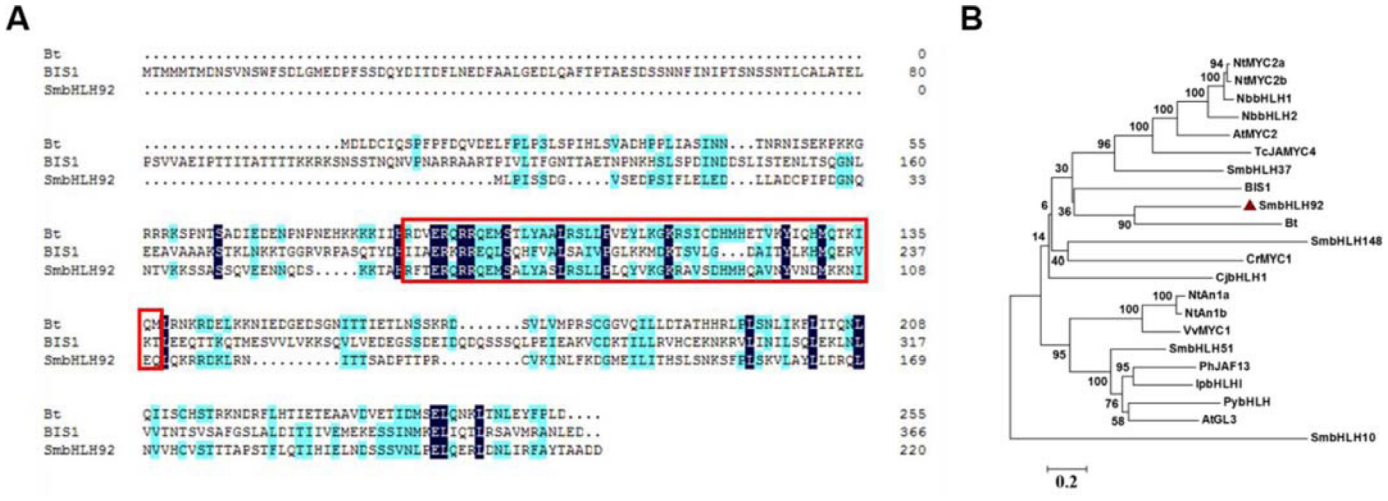

Fig. 1. Sequence analysis of *SmbHLH92*. (A) Multiple alignments of SmbHLH92 with related bHLH proteins BIS1 and Bt of other plant species. The predicted HLH domain is shown in a box with a red line. (B) Phylogenetic analysis of SmbHLH92. Based on the amino acid sequences of *A. thaliana, C. roseus*, and *N. tabacum*, a phylogenetic tree was constructed on MEGA6.0 by using NJ. A bootstrap value of 1000 copies was obtained.

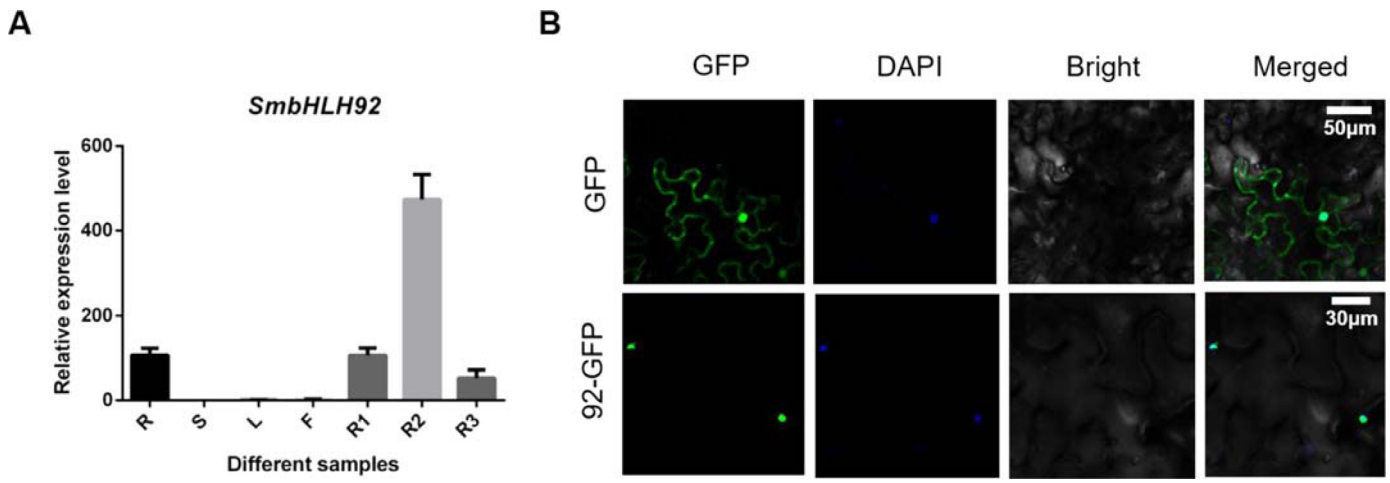

Fig. 2. Expression analysis of *SmbHLH92* in *S. miltiorrhiza*. (A) Expression pattern of *SmbHLH92* in different organs/tissues of *S. miltiorrhiza*. These organs/tissues include root (R), stem (S), leaf (L), flower (F), periderm (R1), phloem (R2), and xylem (R3). (B) Subcellular localization of SmbHLH92 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.

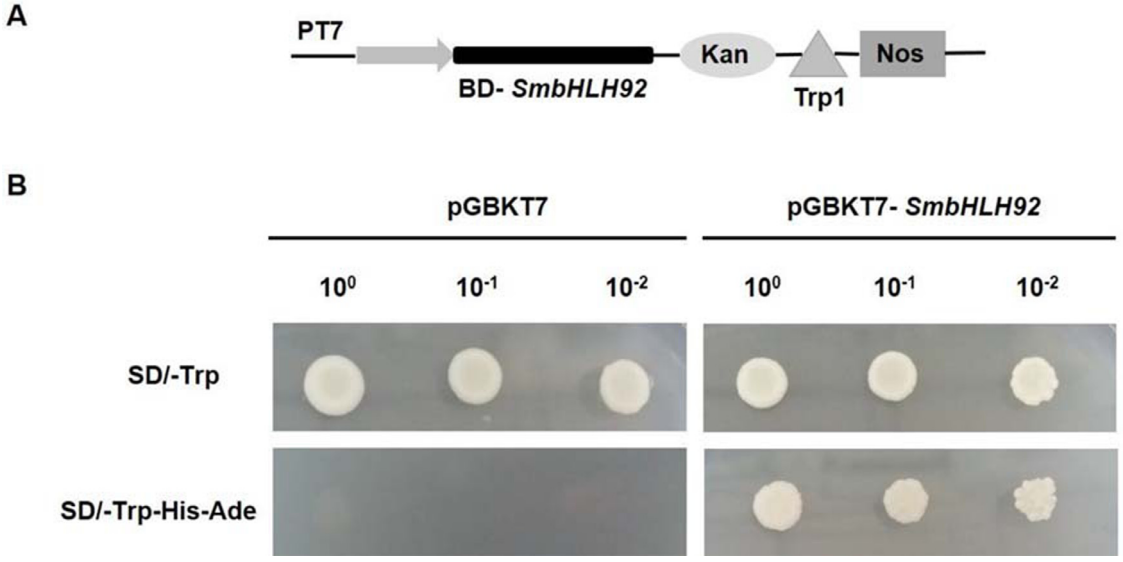

Fig. 3. Transcriptional activity of SmbHLH92. (A) Vector of pGBKT7- *SmbHLH92*. (B) Transcriptional activity of SmbHLH92 in yeast. 10°, 10^{-1}, and 10^{-2} represent the dilution concentrations of the yeast strains, respectively.

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 revealed 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 pGBKT7-*SmbHLH92* and analyzed the transcriptional activity of SmbHLH92 in *AH109* yeast strain (Fig. 3A). The results showed that the yeast containing pGBKT7-*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

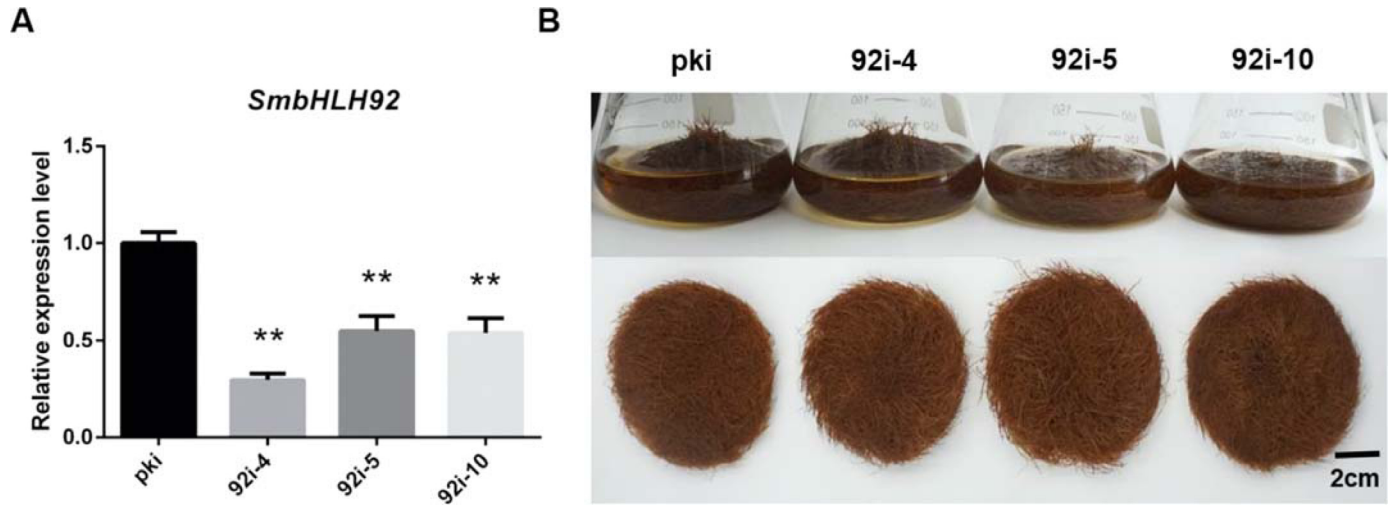

Fig. 4. Expression of *SmbHLH92* in *S. miltiorrhiza* hairy root transgenic lines. (A) Relative quantitative analysis of *SmbHLH92* expression in transgenic lines and control of *S. miltiorrhiza* hairy roots. The results were analyzed using the $2^{-\Delta\Delta CT}$ method. *SmACTIN* is used as an internal 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.

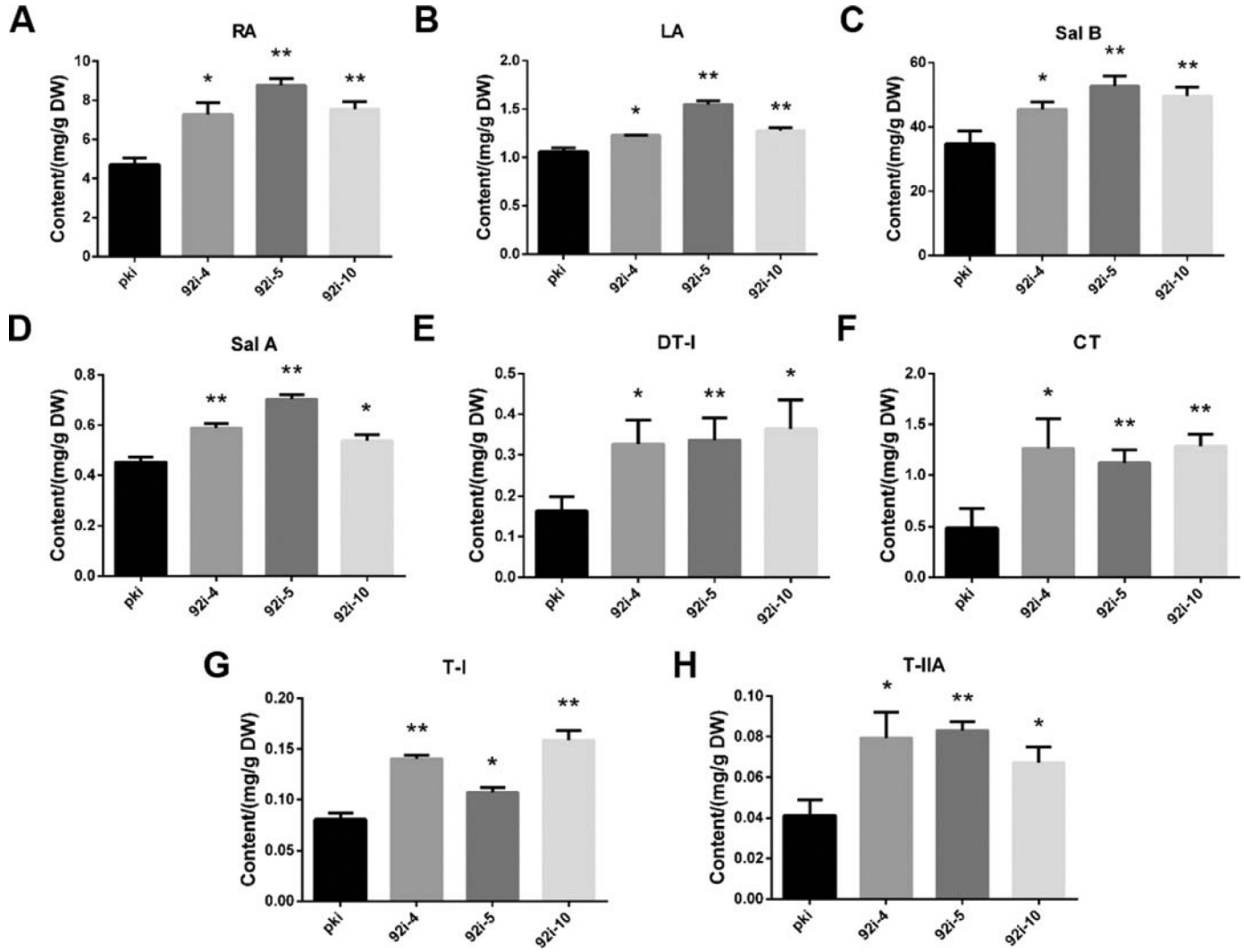

Fig. 5. Content of phenolic acid and tanshinone in hairy roots of *SmbHLH92*-RNAi. (A)–(D) Contents of RA (A), LA (B), Sal B (C), and Sal A (D) in the transgenic and control hairy roots of *S. miltiorrhiza*. (E)–(H) Contents of DT-I (E), CT (F), T-I (G), and T-IIA (H) in hairy roots of transgenic and 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

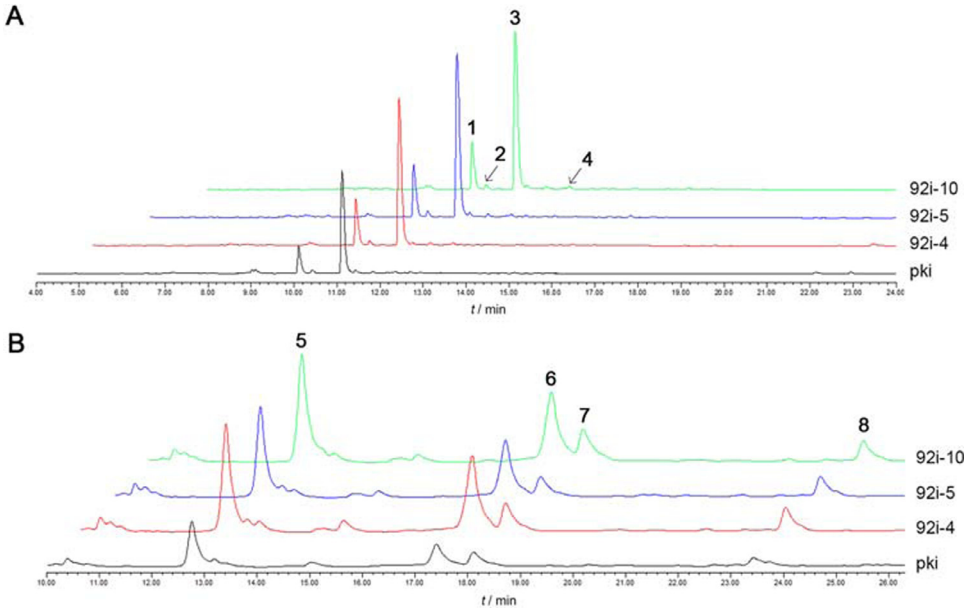

Fig. 6. Typical UPLC curves for phenolic acid and tanshinone. (A) Typical UPLC spectrum of phenolic acid in hairy roots of *SmbHLH92*-RNAi. The four main compounds are labeled 1–4, identified as RA, LA, Sal B, and Sal A. (B) Typical UPLC spectrum of tanshinone in hairy roots of *SmbHLH92*-RNAi. The four main compounds are labeled 5–8, identified as DT-I, CT, T-I, and T-IIA.

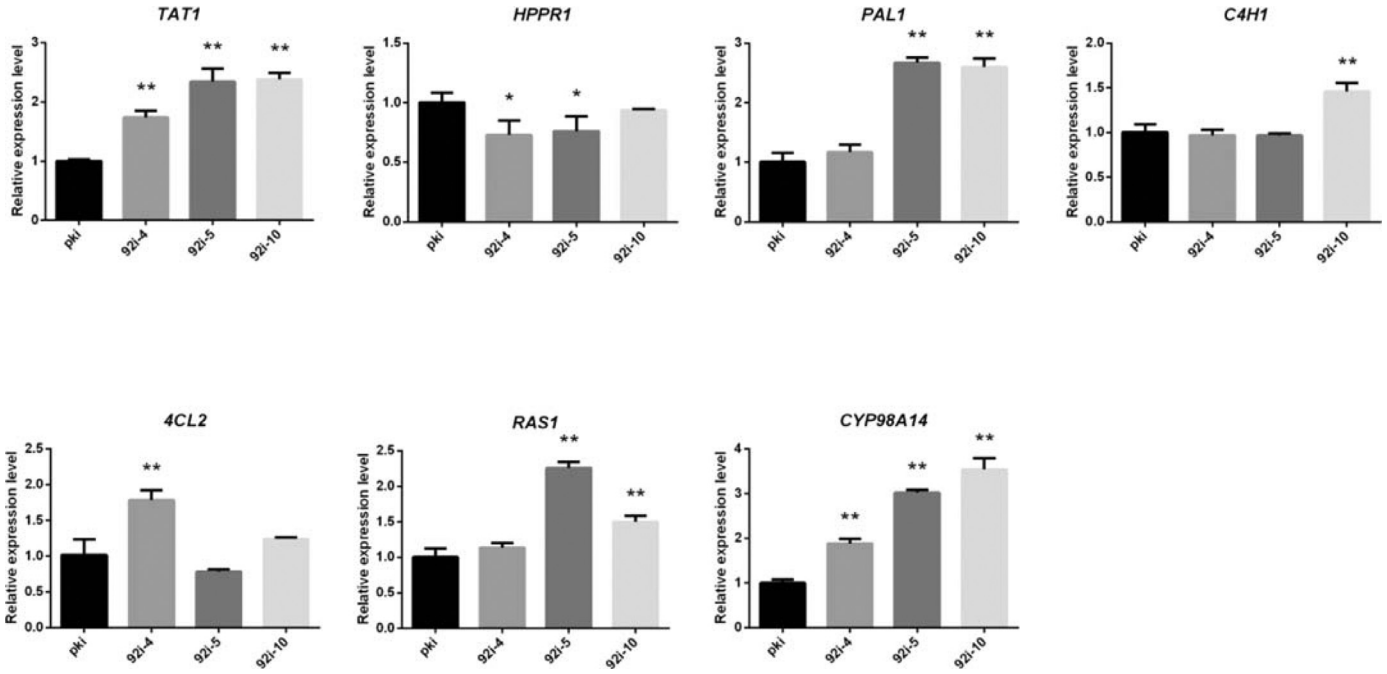

Fig. 7. Relative expression levels of phenolic acid biosynthetic pathway genes in hairy root transgenes and control lines. All values are expressed as mean ± SD. Statistical significance is assessed by Student's *t*-test (n.s.: $P > 0.05$; *: $0.01 < P < 0.05$; **: $P < 0.01$).

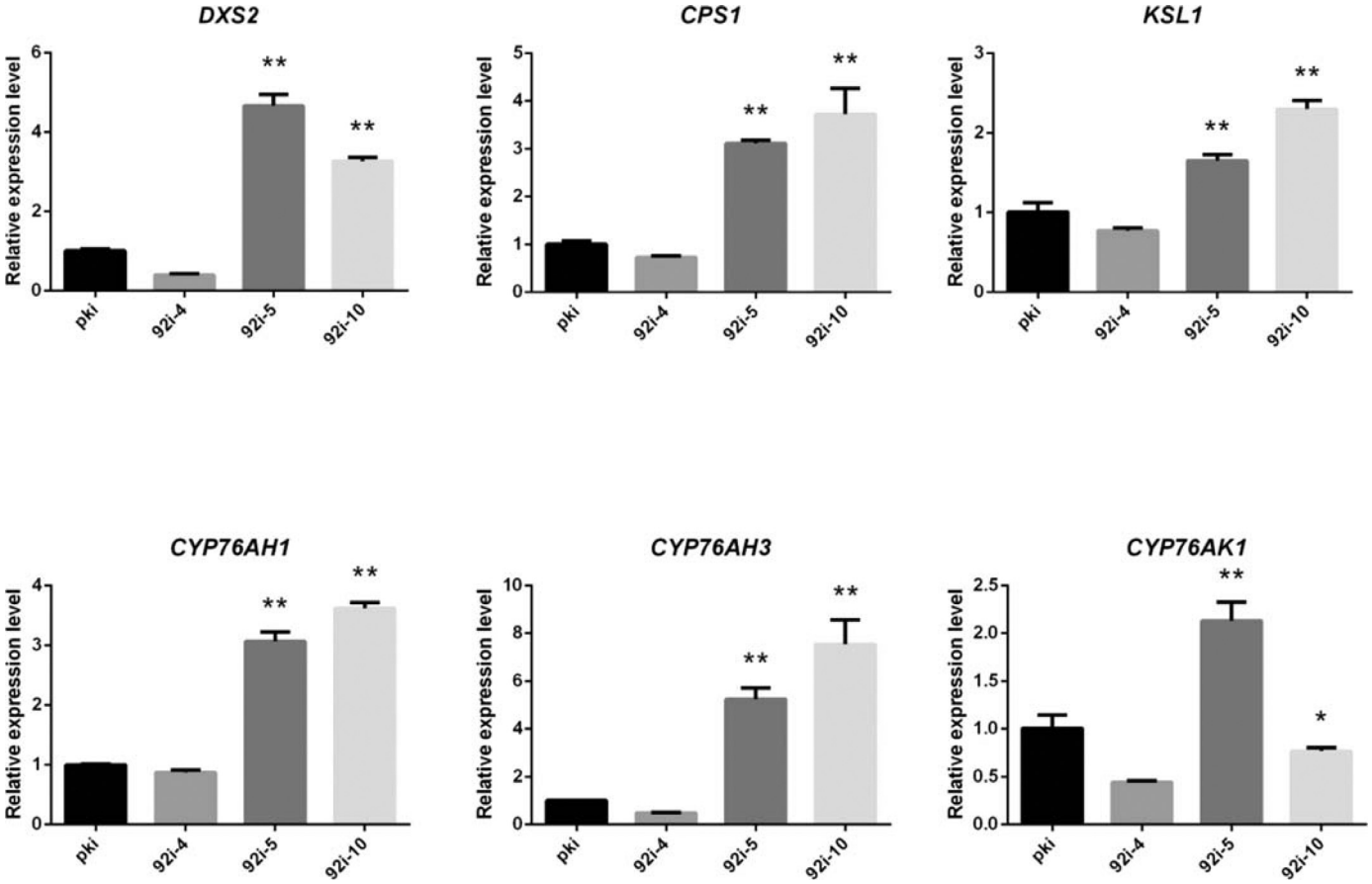

Fig. 8. Relative expression levels of tanshinone biosynthetic pathway genes in hairy root transgenes and control lines. All values are expressed as means ± SD. Statistical significance is assessed with Student's *t*-test (n.s.: $P > 0.05$; *: $0.01 < P < 0.05$; **: $P < 0.01$).

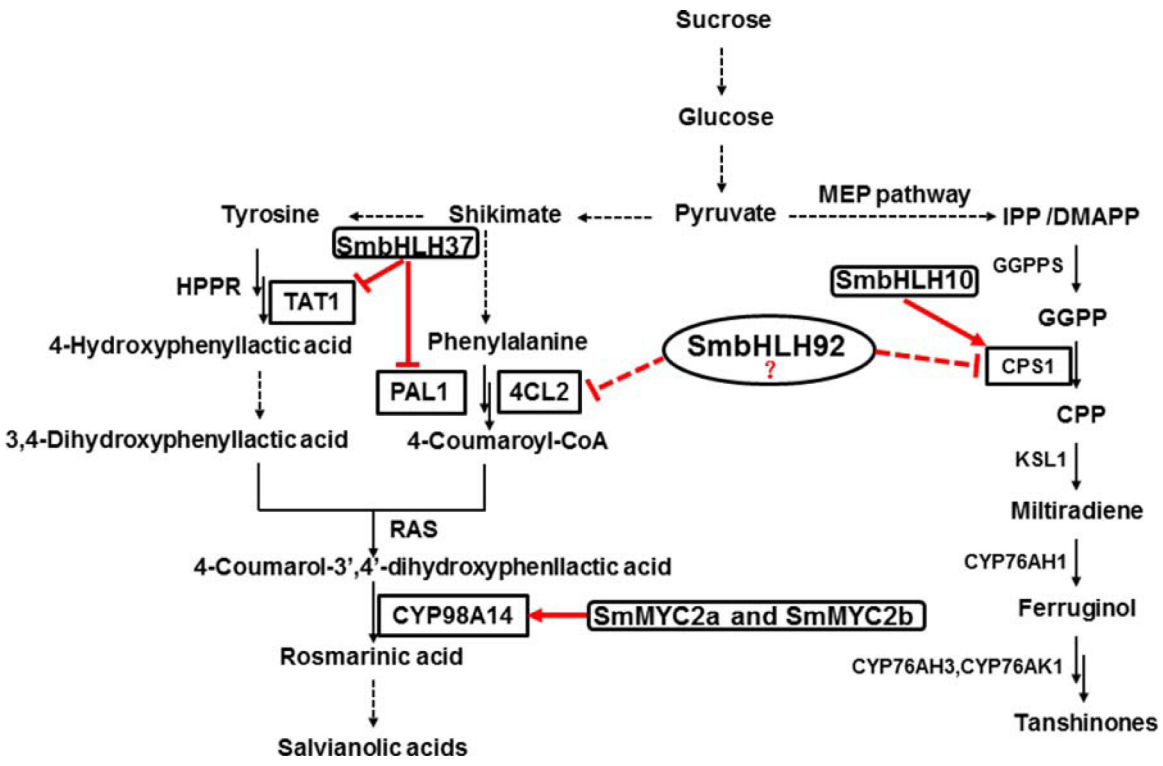

Fig. 9. Predicted work model for bHLH transcription factors in *S. miltiorrhiza*.

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, CYP76AH1* and *CYP76AH3* 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 (Y1H) method. However, Y1H 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 SmbHLH37 were *CYP98A14* (Zhou et al., 2016), *CPS1, CPS5 and DXS2* (Xing et al., 2018b), and *TAT1* and *PAL1* (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 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*. 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.

Acknowledgments

This work was supported by the National Natural Science Foundation (Grant No. 31570302, 81973422) and Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Sciences (CIFMS, 2016-I2M-3–016).

Supplementary materials

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

References

Dong, Y., Wang, C. P., Han, X., Tang, S., Liu, S., & Xia, X. L. (2014). A novel bHLH transcription factor *PebHLH35* from *Populus euphratica* confers drought tolerance through regulating stomatal development, photosynthesis and growth in *Arabidopsis*. *Biochemical and Biophysical Research Communications, 450*(1), 453–458.

Du, T. Z., Niu, J. F., Su, J., Li, S. S., Guo, X. R., Li, L., et al. (2018). SmbHLH37 functions antagonistically with SmMYC2 in regulating jasmonate-mediated biosynthesis of phenolic acids in *Salvia miltiorrhiza*. *Frontiers in Plant Science, 9*, 1720.

Ezer, D., Shepherd, S. J. K., Brestovitsky, A., Dickinson, P., Cortijo, S., Charoensawan, V., et al. (2017). The G-Box transcriptional regulatory code in *Arabidopsis*. *Plant Physiology, 175*(2), 628–640.

Feller, A., Machemer, K., Braun, E. L., & Grotewold, E. (2011). Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant Journal, 66*(1), 94–116.

Gajewska, P., Janiak, A., Kwasniewski, M., Kedziorski, P., & Szarejko, I. (2018). Forward genetics approach reveals a mutation in bHLH transcription factor-encodinggene as the best candidate for the root hairless phenotype in barley. *Frontiers in Plant Science, 9*, 1229.

Gonzalez, A., Zhao, M., Leavitt, J. M., & Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. *Plant Journal, 53*(5), 814–827.

Goossens, J., Mertens, J., & Goossens, A. (2017). Role and functioning of bHLH transcription factors in jasmonate signalling. *Journal of Experimental Botany, 68*(6), 1333–1347.

Ho, H. C., & Hong, C. Y. (2011). Salvianolic acids: Small compounds with multiple mechanisms for cardiovascular protection. *Journal of Biomedical Science, 18*(1), 30 -30.

Hong, G. J., Xue, X. Y., Mao, Y. B., Wang, L. J., & Chen, X. Y. (2012). Arabidopsis MYC2 interacts with Della proteins in regulating sesquiterpene synthase gene expression. *The Plant cell, 24*(6), 2635–2648.

Kumar, S., Tamura, K., Jakobsen, I. B., & Nei, M. (2001). MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics, 17*(12), 1244–1245.

Li, Y. G., Song, L., Liu, M., Hu, Z. B., & Wang, Z. T. (2009). Advancement in analysis of *Salviae miltiorrhizae* Radix et Rhizoma (Danshen). *Journal of Chromatography A, 1216*(11), 1941–1953.

Li, Z. M., Xu, S. W., & Liu, P. Q. (2018). *Salvia miltiorrhiza* Burge (Danshen): A golden herbal medicine in cardiovascular therapeutics. *Acta Pharmacologica Sinica, 39*(5), 802–824.

Liu, Z. J., Zhang, Y. Q., Wang, J. F., Li, P., Zhao, C. Z., & Chen, Y. D. (2015). Phytochrome-interacting factors PIF4 and PIF5 negatively regulate anthocyanin biosynthesis under red light in *Arabidopsis* seedlings. *Plant Science, 238*, 64–72.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods (San Diego, Calif.), 25*(4), 402–408.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406–425.

Shang, Y., Ma, Y. S., Zhou, Y., Zhang, H. M., Duan, L. X., & Chen, H. M. (2014). Plant science. Biosynthesis, regulation, and domestication of bitterness in cucumber. *Science (New York, N.Y.), 346*(6213), 1084–1088.

Shi, M., Luo, X. Q., Ju, G. H., Li, L. L., Huang, S. X., Zhang, T., et al. (2016). Enhanced diterpene tanshinone accumulation and bioactivity of transgenic *Salvia miltiorrhiza* hairy roots by pathway engineering. *Journal of Agricultural and Food Chemistry, 64*(12), 2523–2530.

Todd, A. T., Liu, E., Polvi, S. L., Pammett, R. T., & Page, J. E. (2010). A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in *Nicotiana benthamiana*. *Plant Journal, 62*(4), 589–600.

Van Moerkercke, A., Steensma, P., Schweizer, F., Pollier, J., Gariboldi, I., Payne, R., et al. (2015). The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. *Proceedings of the National Academy of Sciences of The United States of America, 112*(26), 8130–8135.

Wang, B., Sun, W., Li, Q. S., Li, Y., Luo, H. M., & Song, J. Y. (2015). Genome-wide identification of phenolic acid biosynthetic genes in *Salvia miltiorrhiza*. *Planta, 241*(3), 711–725.

Wang, X. H., Morris-Natschke, S. L., & Lee, K. H. (2007). New developments in the chemistry and biology of the bioactive constituents of Tanshen. *Medicinal Research Reviews*, *27*(1), 133–148.

Wu, Y. C., Zhang, Y., Li, L., Guo, X. R., Wang, B., & Cao, X. Y. (2018). AtPAP1 interactswith and activates SmbHLH51, a positive regulator to phenolic acids biosynthesis in *Salvia miltiorrhiza*.. *Frontiers in Plant Science, 9*, 1687.

Xin, T. Y., Zhang, Y., Pu, X. D., Gao, R. R., Xu, Z. C., & Song, J. Y. (2019). Trends in herbgenomics. *Science China-Life Sciences, 62*(3), 288–308.

Xing, B. C., Liang, L. J., Liu, L., Hou, Z. N., Yang, D. F., & Yan, K. J. (2018a). Overexpression of SmbHLH*148* induced biosynthesis of tanshinones as well as phenolic acids in *Salvia miltiorrhiza* hairy roots. *Plant Cell Reports, 37*(12), 1681–1692.

Xing, B. C., Yang, D. F., Yu, H. Z., Zhang, B. X., Yan, K. J., & Zhang, X. M. (2018b). Overexpression of SmbHLH*10* enhances tanshinones biosynthesis in *Salvia miltiorrhiza* hairy roots. *Plant Science, 276*, 229–238.

Xu, W. R., Zhang, N. B., Jiao, Y. T., Li, R. M., Xiao, D. M., & Wang, Z. P. (2014). The grapevine basic helix-loop-helix (bHLH) transcription factor positively modulates CBF-pathway and confers tolerance to cold-stress in *Arabidopsis*. *Molecular Biology Reports, 41*(8), 5329–5342.

Xu, Z. C., Peters, R. J., Weirather, J., Luo, H. M., Liao, B. S., Zhang, X., et al. (2015). Full-length transcriptome sequences and splice variants obtained by a combination of sequencing platranscription factororms applied to different root tissues of *Salvia miltiorrhiza* and tanshinone biosynthesis. *Plant Journal, 82*(6), 951–961.

Zhang, X., Luo, H. M., Xu, Z. C., Zhu, Y. J., Ji, A. J., & Song, J. Y. (2015). Genome-wide characterisation and analysis of bHLH transcription factors related to tanshinone biosynthesis in *Salvia miltiorrhiza*. *Scientific Reports, 5*, 11244.

Zhang, Y., Jiang, P. X., Ye, M., Kim, S.-. H., Jiang, C., & Lü, J. X. (2012). Tanshinones: Sources, pharmacokinetics and anti-cancer activities. *International Journal of Molecular Sciences, 13*(10), 13621–13666.

Zhang, Y., Yan, Y. P., & Wang, Z. Z. (2010). The Arabidopsis PAP1 transcription factor plays an important role in the enrichment of phenolic acids in *Salvia miltiorrhiza*. *Journal of Agricultural and Food Chemistry, 58*(23), 12168–12175.

Zhao, G. R., Zhang, H. M., Ye, T. X., Xiang, Z. J., Yuan, Y. J., & Guo, Z. X. (2008). Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B. *Food and Chemical Toxicology, 46*(1), 73–81.

Zhou, Y. Y., Sun, W., Chen, J. F., Tan, H. X., Xiao, Y., Li, Q., et al. (2016). *SmMYC2a* and *SmMYC2b* played similar but irreplaceable roles in regulating the biosynthesis of tanshinones and phenolic acids in *Salvia miltiorrhiza*. *Scientific Reports, 6*, 22852.