Plant Cell Reports
https://doi.org/10.1007/s00299-018-2339-9

ORIGINAL ARTICLE

Overexpression of *SmbHLH148* induced biosynthesis of tanshinones as well as phenolic acids in *Salvia miltiorrhiza* hairy roots

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Received: 16 June 2018 / Accepted: 23 August 2018

Abstract

Key message **SmbHLH148 activated the whole biosynthetic pathways of phenolic acids and tanshinones, thus upregulated the production of both the two groups of pharmaceutical ingredients in *Salvia miltiorrhiza*.**

Abstract Phenolic acids and tanshinones are the two important groups of pharmaceutical ingredients presented in *Salvia miltiorrhiza* Bunge. The bHLH transcription factors could regulate secondary metabolism efficiently in plants. However, there are only some MYCs have been studied on regulation of either phenolic acids or tanshinones biosynthesis. In this study, a bHLH TF named SmbHLH148, which is homologous to AtbHLH148, AtbHLH147 and CubHLH1, was isolated and functionally characterized from *S. miltiorrhiza*. Transcription of *SmbHLH148* could be intensely induced by ABA and also be moderately induced by MeJA and GA. SmbHLH148 is present in all the six tissues and mostly expressed in fibrous root and flowers. Subcellular localization analysis found that SmbHLH148 was localized in the nucleus. Overexpression of *SmbHLH148* significantly increased not only three phenolic acids components accumulation but also three tanshinones content. Content of caffeic acid, rosmarinic acid and salvianolic acid B were reached to 2.87-, 4.00- and 5.99-fold of the control in the ObHLH148-3, respectively. Content of dihydrotanshinone I, cryptotanshinone, and tanshinone I were also present highest in ObHLH148-3, reached 2.5-, 5.04- and 3.97-fold of the control, respectively. Expression analysis of pathway genes of phenolic acids and tanshinones in transgenic lines showed that most of them were obviously upregulated. Moreover, transcription of *AREB* and *JAZs* were also induced in *SmbHLH148* overexpression lines. These results suggested that SmbHLH148 might be taken part in ABA and MeJA signaling and activated almost the whole biosynthetic pathways of phenolic acids and tanshinones, thus the production of phenolic acids and tanshinones were upregulated.

Keywords bHLH transcription factor · Phenolic acids · Tanshinones · Transgenic · Secondary metabolism · *Salvia miltiorrhiza*

Communicated by Salim Al-Babili.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00299-018-2339-9) contains supplementary material, which is available to authorized users.

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Published online: 18 September 2018

Introduction

The phenolic acids and the tanshinones are two major groups of bioactive ingredients in Danshen (root of S*alvia miltiorrhiza* Bunge), having a range of pharmacological effects, such as anticancer, antioxidant, antibacterial, and anti-inflammatory activities (Zhou et al. 2011; Wang et al. 2015). Danshen has been used mostly for the treatment of cardiovascular and cerebrovascular diseases. The phenolic acids mainly include caffeic acid (CA), rosmarinic acid (RA), and salvianolic acid B (SAB), while the tanshinones mainly include tanshinone I (T-I), cryptotanshinone (CT), dihydrotanshinone I (DT-I), and tanshinone IIA (T-IIA) (Shi et al. 2005; Liu et al. 2006). Because of its pharmacological action and commercial value, numerous studies have focused on improving the production of phenolic acids and tanshinones in *S. miltiorrhiza* using gene engineering, metabolic engineering or fermentation engineering.

The biosynthetic pathways of phenolic acids and tanshinones were both well elucidated. The hydrophilic phenolic acids components are derived via the phenylpropanoid pathway and the tyrosine pathway (Di et al. 2013). Phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), hydroxycinnamate coenzyme A ligase (4CL), tyrosine aminotransferase (TAT), 4-hydroxyphenylpyruvate reductase (HPPR), rosmarinic acid synthase (RAS), and CYP98A14 were identified as the limiting velocity enzymes for phenolic acids biosynthesis (Fig. 1a). The lipophilic tanshinones are biosynthesized via two pathways in plants: the mevalonate (MVA) pathway in cytosol and the methylerythritol phosphate (MEP) pathway in plastids (Gao et al. 2014). And the key enzyme genes of tanshinones biosynthetic pathways including 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), geranylgeranyl diphosphate synthase (GGPPS), copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), and a cytochrome P450 enzyme (CYP76AH1) were also being cloned and analyzed (Fig. 1b). Overexpression of key enzyme gene involved in the biosynthetic pathways or suppress gene expression of competitive pathways could be a viable approach to increase the targeted compounds

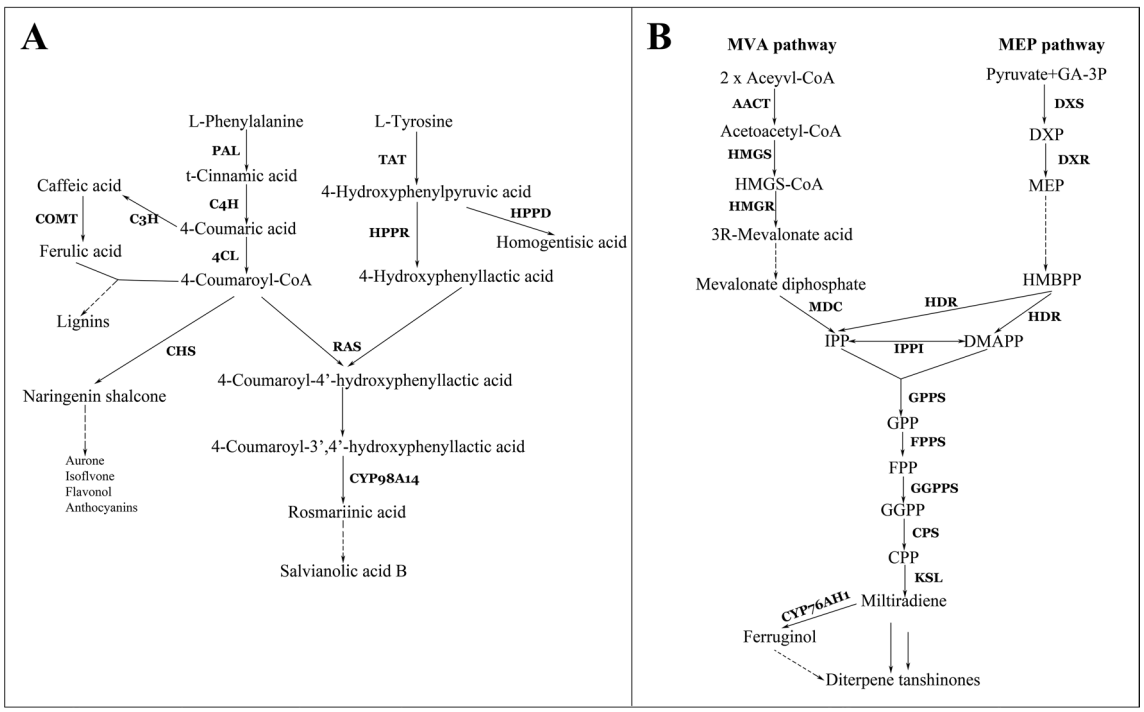

Fig. 1 The metabolic pathways of phenolic acids (**a**) and tanshinones (**b**) in *S. miltiorrhiza*. Multiple enzymatic steps are represented by dotted lines. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamic acid 4-hydroxylase, *4CL* hydroxycinnamate coenzyme A ligase, *HPPR* 4-hydroxyphenylpyruvate reductase, *RAS* rosmarinic acid synthase, *TAT* tyrosine aminotransferase, *CYP98A14* a cytochrome P450-dependent monooxygenase, *AACT* acetyl-CoA C-acetyltransferase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *MK* Mevalonate kinase, *MDC* mevalonate 5-diphosphate decarboxylase, *DXS* 1-deoxy-D-xylulose-5-phosphate synthase, *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase, *HDS* 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, *HDR* 1-hydroxy-2- methyl-2-(E)-butenyl-4-diphosphate reductase, *IPPI* isopentenyl-diphosphate deltaisomerase, *GPPS* geranyl diphosphate synthase, *FPPS* farnesyl diphosphate synthase, *GGPPS* Geranylgeranyl diphosphate synthase, *CPS* copalyl diphosphate synthase, *KSL* Kaurene synthase-like

production (Xiao et al. 2010; Kai et al. 2011; Song and Wang 2011; Cui et al. 2015; Min et al. 2016).

Transcription factors are DNA-binding proteins that could regulate more than one gene transcription at the same time. It has important roles in regulating development, primary and secondary metabolism, abiotic and biotic stress of plants. It could be an effective strategy that use transcription factors to improve the production of pharmaceutically important plant secondary metabolites, such as flavonoids and alkaloids (Gantet and Memelink 2002). The bHLH superfamily proteins is the second largest TF family in plant. And the bHLHs' transcriptional regulation of anthocyanin sharing parts of the phenylpropanoid pathway with phenolic acids was well studied (Yang et al. 2012a, b). *ItIVS* encodes a basic helix–loop–helix protein positive regulate pigments accumulation in flower and seeds of *Ipomoea tricolor* (Park 2012). A bHLH protein, Anthocyanin 1 (An-1), of *Petunia* directly activated transcription of structural anthocyanin genes and induced anthocyanin biosynthesis in the flowers (Koes 2000). The bHLH proteins GL3, EGL3, and TT8 of *Arabidopsis thaliana* forming a transcriptional regulation complex with MYB proteins and WD40 repeat containing protein TTG1, also activated anthocyanin biosynthetic genes (Antonio et al. 2008; Dubos et al. 2010). Overexpression of *CubHLH1* in tomato reduced the lycopene content (Endo et al. 2016). Another important group of bHLH regulator is MYC that could regulate flavones biosynthetic. In *A. thaliana*, AtMYC3 interacts with JAZs to integrate jasmonate (JA) signaling pathways to upregulate the expression of dihydroflavonol reductase (*DFR*) and improved anthocyanin accumulation in seedlings (Cheng et al. 2011).

The bHLHs also showed important roles in the regulation of pharmaceutical terpenoids biosynthesis. The AtMYC2 could increase emission of sesquiterpene by binding to the promoters of sesquiterpene synthase genes *TPS21* and *TPS11* directly and activate their expression (Hong et al. 2012). Another *A. thaliana* bHLH TF, phytochrome-interacting factor 5 (PIF5), positively regulated the MEP pathway and enhanced isopentyl diphosphate (IPP) metabolism (Mannen et al. 2014). In *Artemisia annua*, overexpression of *AaMYC2* significantly activated the transcript levels of *CYP71AV1* and *DBR2*, which resulted in an increased artemisinin content (Shen et al. 2016). Chihiro et al. found a diterpenoid phytoalexin factor (DPF), which belongs to bHLH family, positively regulating diterpenoid phytoalexins (DP) accumulation via transcriptional regulation of DP biosynthetic genes in rice (Yamamura et al. 2015). In *Catharanthus roseus*, CrMYC2 binds to cis-elements in the *ORCA3* promoter directly, thereby controlling terpenoid indole alkaloid accumulation (Zhang et al. 2011).

There are 127 bHLH transcription factor genes in the genome of *S. miltiorrhiza*, and several bHLH genes were calculated as potentially involved in the regulation of tanshinones biosynthesis (Zhang et al. 2015). In *S. miltiorrhiza*, there only have MYCs which being studied on regulation of phenolic acids or tanshinones biosynthesis. For instance, report of Yang et al. demonstrated that overexpression of *SmMYC2* promoted the production of phenolic acids (Yang et al. 2017). Unfortunately, they did not detect neither T-IIA nor CT in their transgenic lines. There is another report on *SmMYC*, which suggested that the accumulation tanshinones and SAB was impaired by the loss of function in SmMYC2a/b (Zhou et al. 2016). However, the opposite effect by overexpression of *SmMYC2a/b* has not been studied yet, and the identification of direct target genes in tanshinones biosynthesis is also awaiting. So far, most of the TFs were taken different responsibility between biosynthesis of phenolic acids and tanshinones, while the bHLH which could upregulate accumulation of both phenolic acids and tanshinones have not been found. And yet, the detailed functions of the large family of transcription factors remain unknown.

In the present study, the SmbHLH148 which is homologous to AtbHLH148, AtbHLH147 and CubHLH1 was obtained. Transcription of *SmbHLH148* could be intensely induced by ABA. Overexpression of *SmbHLH148* could promote both the phenolic acids and the tanshinones accumulation in *S. miltiorrhiza* hairy roots.

Materials and methods

Hairy roots culture

Agrobacterium rhizogenes (ATCC15834) with Ri (root inducing) T-DNA (transfer DNA) was used to infect aseptic leaves of *S. miltiorrhiza*, then the hairy roots of *S. miltiorrhiza* were obtained. Samples of the hairy roots weighing 0.2 g were inoculated into 100 mL beaker flasks contains 50 mL of 6,7-V liquid medium (with 30 $g{\cdot}L^{-1}$ sucrose). The beaker flasks containing the hairy roots were then placed on an orbital shaker at 110 $rpm{\cdot}min^{-1}$, 25 °C in the dark. The hairy roots were harvested after 30 days and used for real-time quantitative PCR analysis, HPLC analysis.

For hormone treatments, 100 μM MeJA (Sigma–Aldrich), 100 μM abscisic acid (ABA; Sigma–Aldrich) and 100 μM gibberellic acid (GA3; Sigma–Aldrich) were used, whereas hairy roots supplemented with 50% DMSO was used as a control. Elicitation was performed on the 18th day after inoculation. Hairy roots were harvested from the culture medium on 0, 1, 2, 4, 8, 12, 24 and 48 h after treatment, respectively. The experiments were performed in triplicate, and the results are means ± SD.

Total RNA and DNA extraction

The total complete RNA was isolated from the frozen *S. miltiorrhiza* hairy roots using the RNAprep Pure Plant Kit (TIANGEN, China). The RNA was then reversely transcribed to generate the first strand cDNA, according to the manufacturer's instructions of the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Japan). To isolate the genomic DNA, an improved cetyltrimethylammonium bromide (CTAB) method was used. The quality and concentration of the genomic DNA and RNA were examined by agarose gel electrophoresis and spectrophotometer analysis (Thermo Scientific NanoDrop 2000).

Bioinformatics analysis of SmbHLH148

We isolated the sequence of *SmbHLH148* from the *S. miltiorrhiza* transcriptome database (Xue et al. 2016) and found the open reading frame (ORF) with OFP-finder (https://www.ncbi.nlm.nih.gov/orffinder/). BLAST was used to determine the differences between *SmbHLH148* sequences and the NCBI database (https://www.ncbi.nlm.nih.gov/). Its conserved domain was identified by BLASTP of NCBI database and searched with SMART server (http://smart.embl-heidelberg.de/). Phylogenetic trees were generated with the MEGA 6 software program by employing the neighbor joining method, with 500 bootstrap replicates. All amino acid sequences of the other species were downloaded from the NCBI database.

Subcellular localization analysis

A vector pTF486 containing the open reading frame of eGFP was used in this study (Liu et al. 2010). The ORF of *SmbHLH148* was amplified with primers SmGFP1-Sal I (5′-ACGCGTCGACATGATGTCATCGCTGTTATCATCGA-3′) and SmGFP2-Nco I (5′-CATGCCATGGCATGACCA CCACCACCACCGTTGGGTGGG GTCCGCAC-3′) using PrimeSTAR® HS DNA Polymerase (Takara, Japan). The amplification sequence was ligated with Sal I and Nco I-digested pTF486 vector to generate a SmbHLH148-GFP fusion construct under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter. The construct was confirmed by sequencing and used for transient transformation of onion epidermis via a gene gun (Bio-Rad, Hercules, CA, USA). After 24 h of incubation, GFP fluorescence in transformed onion cells was observed under a confocal microscope (Nikon A1, Tokyo, Japan).

Construction of plant expression vectors and acquisition of positive transgenic hairy roots

The coding region of *SmbHLH148* was amplified and cloned into the restriction site Xba I and Sac I of the pCAMBIA1300 binary vector under the control of the CaMV35S promoter and the NOS terminator. The recombinant plasmids SmbHLH148-1300 was transformed into *A. rhizogenes* (ATCC15834). The hairy roots lines were acquired from the transformation of leaf explants from the sterile plantlets of *S. miltiorrhiza*. The positive transgenic lines were identified by PCR using *rolB, rolC, HPT* and *SmbHLH148* specific primer. These latter lines were used for RNA and metabolites extraction and they were regularly sub-cultured (every 30 days). All the primers used for the overexpression vector construction and the PCR identification of transgenic lines are listed in Table S1.

Metabolites extraction and HPLC analysis

The dried hairy roots were powdered using a homogenizer (Bioprep-24, Hangzhou, China). The sample powder (20 mg) was extracted with 70% methanol (2 mL) under ultrasonic treatment for 45 min and the resulting mixture was centrifuged at 8000*g* for 10 min. The supernatant was filtered through a 0.45-μm organic membrane filter and analyzed by HPLC. The metabolite contents were determined on a Waters HPLC e2695 system (Waters, Milford, MA, USA) equipped with an automatic sample injector and a Waters 2996 photodiode array detector. Chromatographic separation was performed using a SunFire C18 column (4.6 mm × 250 mm, 5 μm particle size) at 30 °C. Empower 3 software was used for data acquisition and processing. The sample injection volume was 10 μL and the PDA wavelengths used for the detection of the phenolic acids and lipid-soluble diterpenoids were 288 and 270 nm, respectively. Separation was achieved by elution using a linear gradient with solvents A (acetonitrile) and B (0.026% phosphoric acid solution). The gradient was as follows (all concentrations are v/v): 0–10 min, 5–20% A; 10–15 min, 20–25% A; 15–20 min, 25% A; 20–25 min, 25–20% A; 25–28 min, 20–30% A; 28–40 min, 30% A; 40–45 min, 30–45% A; 45–58 min, 45–58% A; 58–67 min, 58–50% A; 67–70 min, 50–60% A; 70–80 min, 60–65% A; 80–85 min, 65–95% A; and 85–95 min, 95% A. Standards of the metabolite compounds were purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

RNA isolation and real-time quantitative PCR analysis

Total RNA was extracted from the *S. miltiorrhiza* hairy roots, according to the method described above. The first strand cDNA for RT-qPCR were synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Tokyo, Japan). Primers used for RT-qPCR are listed at supplement Table S2. The *SmActin* gene was used as reference. Real-time PCR was performed according

to the manufacturer's instruction of SYBR® Premix Ex Taq™ II (TliRNaseH Plus, Takara) using the following protocol: 95 °C, 30 s, 1 cycle; 95 °C, 5 s, 58 °C, 30 s, 40 cycles. The program was performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Massachusetts, USA). Quantification of the genes expression was done with comparative CT method ($2^{-\Delta\Delta CT}$). Experiments were performed in triplicate, and the results were represented by their means ± SD.

Results

Bioinformatics analysis of SmbHLH148

SmbHLH148 contained a 675 bp ORF, encoding a protein of 224 amino acids with a predicted molecular mass of 24.740 kDa. Its GenBank accession number is MH472658. The SMART analysis and multiple alignments of the amino acids of SmbHLH148 revealed that it has a basic-Helix–Loop–Helix domain (117–160 aa) (Fig. 2a). BLAST analysis revealed that SmbHLH148 is similar to *Sesamum indicum* transcription factor bHLH148 (XP_011100747) with 79% identity, *Erythranthe guttata* bHLH148-like transcription factor (XP_012836308) with 78% identity, *Olea europaea* var. sylvestris bHLH148-like transcription factor (XP_022849337) with 65% identity and *Nicotiana attenuata* bHLH148-like transcription factor (XP_016473678) with 58% identity at the amino acid level. To visually examine the evolutionary origins, the SmbHLH148, along with SibHLH148 (XP_011100747.1), EgbHLH148-like (XP_012836308.1), OebHLH148 (XP_022849337.1), CcbHLH (PHU29940.1), EgbHLH148 (XP 012840304.1), SlbHLH148-like (XP_004229017.1), NtbHLH148-like (XP_016473678.1), CsbHLH147 (XP_006488826.1), CubHLH1 (BAU09296.1), HabHLH148-like (XP_022000373.1), and bHLH147 (NP_566567), bHLH148 (NP_566287) of *Arabidopsis thaliana* were used to construct a phylogenetic tree (Fig. 2b). The result showed that SmbHLH148 is most closely related to EgbHLH148 from *Erythranthe guttata*, and the identity between SmbHLH148 with the AtbHLH147, AtbHLH148 are 54 and 52%, respectively.

SmbHLH148 is a nuclear-localized protein

Subcellular localization of the protein could help reveal its potential function. The GFP transient expression in onion epidermis was used to examine the subcellular localization of SmbHLH148. Results showed that GFP fluorescence of the control existed in the nucleus and cytoplasm. The GFP fluorescence of SmbHLH148 was dispersive in the nucleus (Fig. 3).

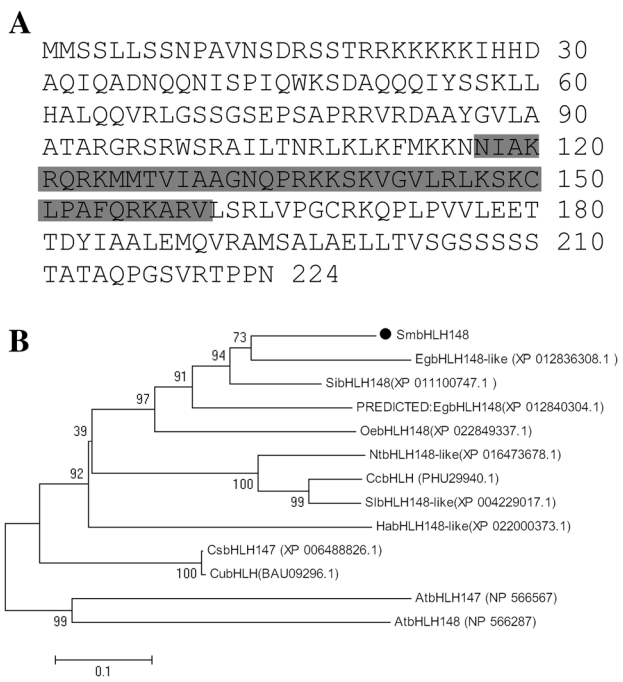

Fig. 2 Sequence analysis of SmbHLH148. **a** Deduced amino acid sequence of SmbHLH148. Predict HLH domain was present in shaded area. **b** Phylogenetic analysis of SmbHLH148. A phylogenetic tree constructed based on the amino acid sequences of SmbHLH148 and SibHLH148 (XP_011100747), EgbHLH148-like (XP_012836308), OebHLH148 (XP_022849337), CcbHLH (PHU29940), SlbHLH148-like (XP_004229017), EgbHLH148 (XP_012840304), NtbHLH148-like (XP_016473678), CsbHLH147 (XP_006488826), CubHLH (BAU09296), HabHLH148-like (XP_022000373), AtbHLH147 (NP_566567), and AtbHLH148 (NP_566287). These phylogenetic trees were constructed via MEGA6, using the neighbor-joining method with 500 bootstrap replicates

Tissue-specific expression and induction pattern of *SmbHLH148*

SmbHLH148 expression in different tissues (stem, leaf, flower, root epidermis, xylem and fibrous root) of two-year-old flowering *S. miltiorrhiza* were performed. The result showed that *SmbHLH148* expressed in all the six tissues of *S. miltiorrhiza*, with highest expression in fibrous root and lowest expression in xylem (Fig. 4a).

To explore whether *SmbHLH148* responded to the phytohormones, the expression of *SmbHLH148* after exogenous hormone treatment was analyzed by RT-qPCR at different time points. It was found that MeJA, ABA and GA induced the *SmbHLH148* expression to varying degrees (Fig. 4b–d). The transcript level of *SmbHLH148* increased to 9.81-fold of the control rapidly after 1 h and kept increasing to 25.46-fold of the control at 8 h after ABA treatment. After treatment with GA, *SmbHLH148* increased to 1.32-fold of the control after 2 h, reached the peak (1.62-fold of the control) at 12 h

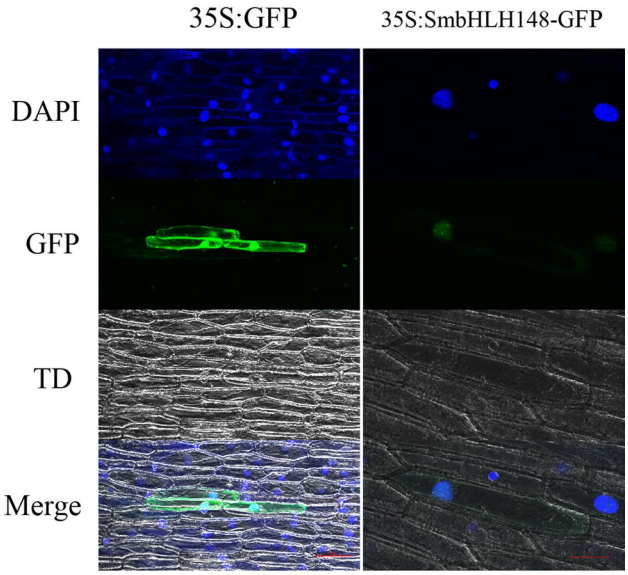

Fig. 3 Subcellular localization of SmbHLH148 protein in onion epidermal cells. Fluorescence was observed using a confocal laser scanning microscope at 24 h after incubation. The pictures show bright field (TD), green fluorescent field (GFP), DAPI and overlay of three fields (merge). The numerical reading of red ruler is 100 μm

and then decreased. After treatment by MeJA, *SmbHLH148* transcripts increased at 1 h, reached the highest level at 4 h (1.71-fold of the control), and then declined back to the base level. These results indicated that *SmbHLH148* was more sensitive to ABA than to GA and MeJA.

Generation of *SmbHLH148*-overexpression hairy roots

In the present study, six transgenic hairy roots lines were obtained through hygromycin resistance screening and PCR selection (Fig. 5a). Relative expression level of *SmbHLH148* in all the transgenic hairy root lines was analyzed with RT-qPCR. Among them, three overexpression lines (ObHLH148-3, ObHLH148-4, ObHLH148-6) presented high expression level of *SmbHLH148*. Transcripts level of *SmbHLH148* in the three overexpressing lines (ObHLH148-3, ObHLH148-4, ObHLH148-6) were approximately 45.50-, 27.62- and 23.03-fold with respect to the control (the line that infect by *A. rhizogenes* directly), respectively (Fig. 5b).

SmbHLH148 promotes both phenolic acids and tanshinones biosynthesis in *S. miltiorrhiza* hairy roots

Compared with the control, accumulation of phenolic acids such as CA, RA and SAB increased in *SmbHLH148*-overexpression hairy roots lines. Content of CA reached to 0.43 mg/g DW in ObHLH148-3 and ObHLH148-6, 2.87-fold of control. The RA content had increased by 300% (5.28 mg/g DW) in ObHLH148-3 when compared to the control. And content of SAB was improved to sixfold (8.30 mg/g DW) of the control in ObHLH148-3 (Fig. 6a). To examine the effect of SmbHLH148 on tanshinones biosynthesis in *S. miltiorrhiza*, content of DT-I, CT, T-I and T-II A were determined. Results showed that SmbHLH148 induced three of the tanshinones ingredients accumulation, except for T-II A. The three major tanshinones contents were highest in OE-SmbHLH148-3, reached 2.5-(DT-I), 5.04-(CT) and 3.97-fold (T-I) of the control, respectively (Fig. 6b). These results indicated that SmbHLH148 could promote both phenolic acids and tanshinones biosynthesis in *S. miltiorrhiza* hairy roots.

SmbHLH148 upregulate expression of pathway genes involved in phenolic acids and tanshinones biosynthetic

To further uncover the function of SmbHLH148 on phenolic acids and tanshinones accumulation, we analyzed the main enzyme genes' expression of their biosynthetic pathways by quantitative RT-PCR. Overexpression of *SmbHLH148* activated genes of the phenylpropanoid pathway (*PAL1, C4H1*), tyrosine pathway (*TAT, HPPR*) and downstream pathway (*RAS, CYP98A14*), except for *4CL1* and *4CL2* (Fig. 7a, b). For tanshinones biosynthetic pathways, including *DXS2* and *DXR* of MEP pathway, *HMGRs* of MVA pathway, the *GGPPS, CPS1, CPS5, KSL1* and *CYP76AH1* of downstream pathway, were all upregulated in *SmbHLH148* overexpression lines compared with those in the control (Fig. 7c–e). Transcript level of *PAL1, C4H1, HPPR, RAS* and *CYP98A14* were 2.91-, 3.13-,1.46-, 2.54-, 3.36-fold higher in ObHLH148-3 than in control, respectively. Expression of *TAT* was induced about 2.94-fold of the control in ObHLH148-4. Expression of *DXS2, DXR, GGPPS, CPS1, CPS5, KSL1* and *CYP76AH1* were also highest in line ObHLH148-3, reaching 8.62-, 2.3-, 2.54-, 5.75-, 6.63-, 1.99- and 4.6-fold of the control, respectively. While three *HMGR*s expression were highest in line ObHLH148-4, 1.41-, 1.64- and 1.54-fold of the control, respectively. The variations of these pathway genes transcription were generally consistent with the active ingredients contents.

Discussion

Phylogenetic analysis result revealed that SmbHLH148 showed high homologous to bHLH148 or bHLH147 proteins of some other species (e.g., OsbHLH148, AtbHLH147, AtbHLH148, CubHLH1). OsbHLH148 from rice, which involved in abiotic stress responses mediated by MeJA and ABA, could be induced by MeJA or ABA (Seo et al. 2011).

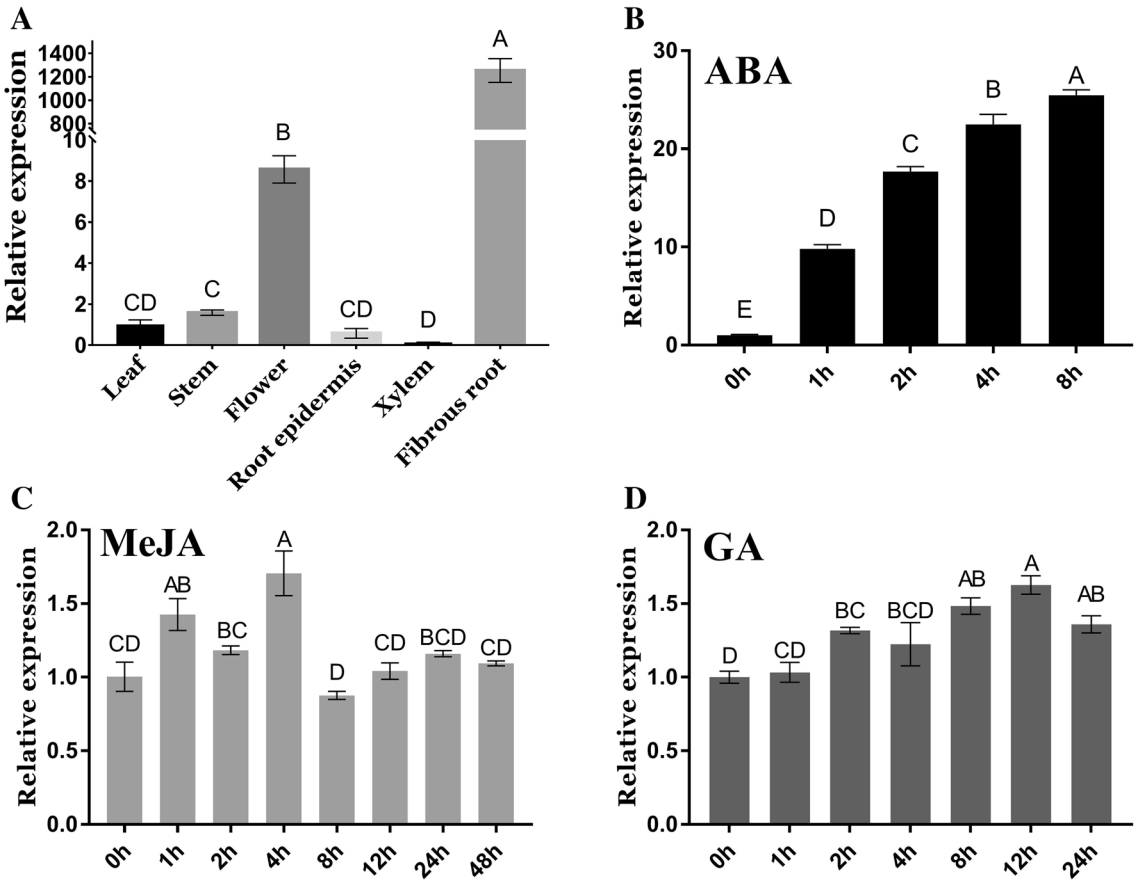

Fig. 4 **a** Expression pattern of *SmbHLH148* in different tissues of *S. miltiorrhiza*. **b** Time course of the expression level of *SmbHLH148* after ABA treatment. **c** The expression level of *SmbHLH148* after MeJA treatment for selected time points. **d** The expression level of *SmbHLH148* after GA treatment for selected points. The results were analyzed using the comparative Ct method. The *S. miltiorrhiza Actin* gene was used as an internal control to normalize expression levels. Bars are means ± SD from three independent biological replicates. One-way ANOVA (followed by a Turkey comparison) tested for significant differences among the means (indicated by different letters at $P < 0.01$)

CubHLH1, a homolog bHLH to SmbHLH148, that was reported could modulate carotenoid metabolism and induce ABA content in transgenic tomato (Endo et al. 2016). In this study, results showed that expression of *SmbHLH148* was significantly induced by ABA, while induced by MeJA, GA were much weaker (Fig. 4b–d). Expression pattern showed that *SmbHLH148* expressed in all the six tissues of *S. miltiorrhiza*, with highest expression in fibrous root and lowest expression in xylem. It's expression pattern was consistent with the position of phenolic acids and tanshinones accumulation. The bioactive components of *S. miltiorrhiza* were mainly present in the root (include fibrous roots). And they were also being detected in leaves, flowers and stem. Our previous study reviews that content of tanshinones in fibrous roots were higher than main root. Previous reports indicated that exogenous ABA could promote the accumulation of phenolic acids (Liang et al. 2013). Moreover, ABA also increased tanshinones production in *S. miltiorrhiza* hairy roots (Yang et al. 2012a, b). AREBs, the ABA-responsive element (ABRE)-binding proteins, which were highly inducible by exogenous ABA and its overexpression increased the contents of RA and SAB in *S. miltiorrhiza* hairy roots (Jia et al. 2017). These indicated that SmbHLH148 could be a candidate regulator of phenolic acids or tanshinones biosynthesis. As the results showed both phenolic acids and tanshinones accumulation were increased in the *SmbHLH148* overexpression lines (Fig. 6). The highest content of the phenolic acids and the tanshinones were observed in transgenic ObHLH148-3, which showed highest *SmbHLH148* expression among transgenic lines. RA content had increased to fourfold of the control (5.28 mg/g DW), and content of SAB was improved to sixfold (8.30 mg/g DW) of the control in ObHLH148-3. Except for T-II A, the other three major tanshinones contents were also highest in OE-SmbHLH148-3, reached 0.39 mg/g DW (DT-I), 1.26 mg/g DW (CT), 12.97 mg/g DW (T-I), respectively.

The key enzyme genes expression of the phenolic acids and tanshinones biosynthetic pathways were analyzed in

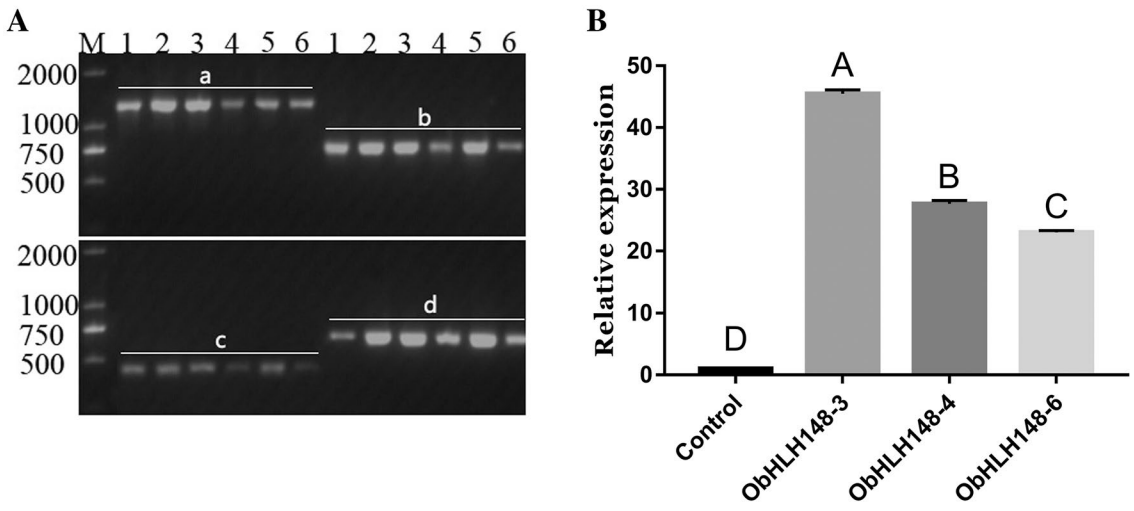

Fig. 5 **a** Identification of positive transgenic hairy root lines by PCR (**a** 35S + SmbHLH148; **b** hptII; **c** rolb; **d** rolc). Numbers above represent individual transgenic lines and M represent DL2000 DNA marker. **b** Relative quantitative analysis of *SmbHLH148* expression in transgenic lines and control of *S. miltiorrhiza* hairy roots. The results were analyzed using the comparative Ct method and presented as foldchanges compared with the control sample. The *S. miltiorrhiza Actin* gene was used as an internal control to normalize expression levels. Bars are means ± SD from three independent biological replicates. One-way ANOVA (followed by a Turkey comparison) tested for significant differences among the means (indicated by different letters at $P < 0.01$)

transgenic lines. Results showed that overexpression of *SmbHLH148* could promote the transcript level of *PAL1, C4H1, TAT, HPPR, RAS* and *CYP98A14*, which involved in phenolic acids biosynthesis (Fig. 7a, b). And the transcript level of *DXS2, DXR, HMGR, GGPPS, CPS1, CPS5, KSL1* and *CYP76AH1* were also being induced in *SmbHLH148* overexpression lines (Fig. 7c–e). These genes play important role in phenolic acids or tanshinones biosynthesis. RNAi of the *SmPAL1* caused a reduction of RA biosynthesis in *S. miltiorrhiza* (Song and Wang 2011). Otherwise, overexpression of *C4H, TAT* or *HPPR* enhanced the production of RA and LAB (Xiao et al. 2010). And the accumulation of RA and SAB obviously decreased in *RAS* or *CYP98A14* antisense transgenic *S. miltiorrhiza* hairy root lines (Di et al. 2013). For the genes of tanshinones biosynthesis, overexpression of *GGPPS* and/or *HMGR* as well as *DXS* in hairy roots can significantly enhance the production of tanshinones (Kai et al. 2011). Furthermore, the overexpression of *CPS1* could enhance tanshinones accumulation, otherwise, silencing of *CPS1* caused a decrease in tanshinones levels and silencing of *CYP76AH1* and *KSL1* also significantly decreased the content of tanshinones (Cheng et al. 2014; Cui et al. 2015; Ma et al. 2016). There are studies suggested that ABA treatment induced activities of PAL and TAT to improve accumulation of phenolic acids (Liang et al. 2013). Besides, ABA treatment upregulated expressions of *HMGR, DXR* and *DXS* (Yang et al. 2012a, b). The ABRE protein, which was induced by ABA treatment, also presented higher transcription in *SmbHLH148* overexpression lines (Fig. S1). Unfortunately, SmbHLH148 could not interact with the ABRE protein that we got in yeast (result not shown). As a family, there are many other ABRE members in *S. miltiorrhiza*. Moreover, some other protein families, like sucrose non-fermenting 1 (SNF1)-related protein kinase 2 (SnRK2), also respond to ABA. And it has been reported that overexpressing SnRK2 increased RA and SAB biosynthesis in the transgenic *S. miltiorrhiza* hairy roots (Jia et al. 2017). The results implied that SmbHLH148 might be directly involved in processes of the inducing phenolic acids and tanshinones metabolic by ABA. The SmbHLH148 induced almost all of the phenolic acids and the tanshinones biosynthetic pathway genes. This indicated that SmbHLH148 might be not only involved in response to ABA, but also other stresses. MeJA treatment induced the transcription of almost all of the phenolic acids pathway genes and then promoted the RA and SAB accumulation (Xing et al. 2018). In addition, the expressions of most investigated genes in tanshinones biosynthetic pathways were up-regulated by MeJA (Kai et al. 2012). Otherwise, GA treatment also could improve the tanshinones accumulation in *S. miltiorrhiza* hairy roots and induce the expression of *CPS* and *KSL* (Bai et al. 2017). The transcription of jasmonate zim-domain transcription factor (JAZ), which is the key components in JA signal processes (Pei et al. 2018), was also promoted by overexpression of *SmbHLH148* (Fig. S2). It is possible that SmbHLH148 played some weak role in the GA and JA signaling. These results implied that SmbHLH148 might not be simply took part in the processes that ABA regulate the accumulation of phenolic acids and tanshinones but also MeJA signaling. And SmbHLH148 activated almost the whole biosynthetic

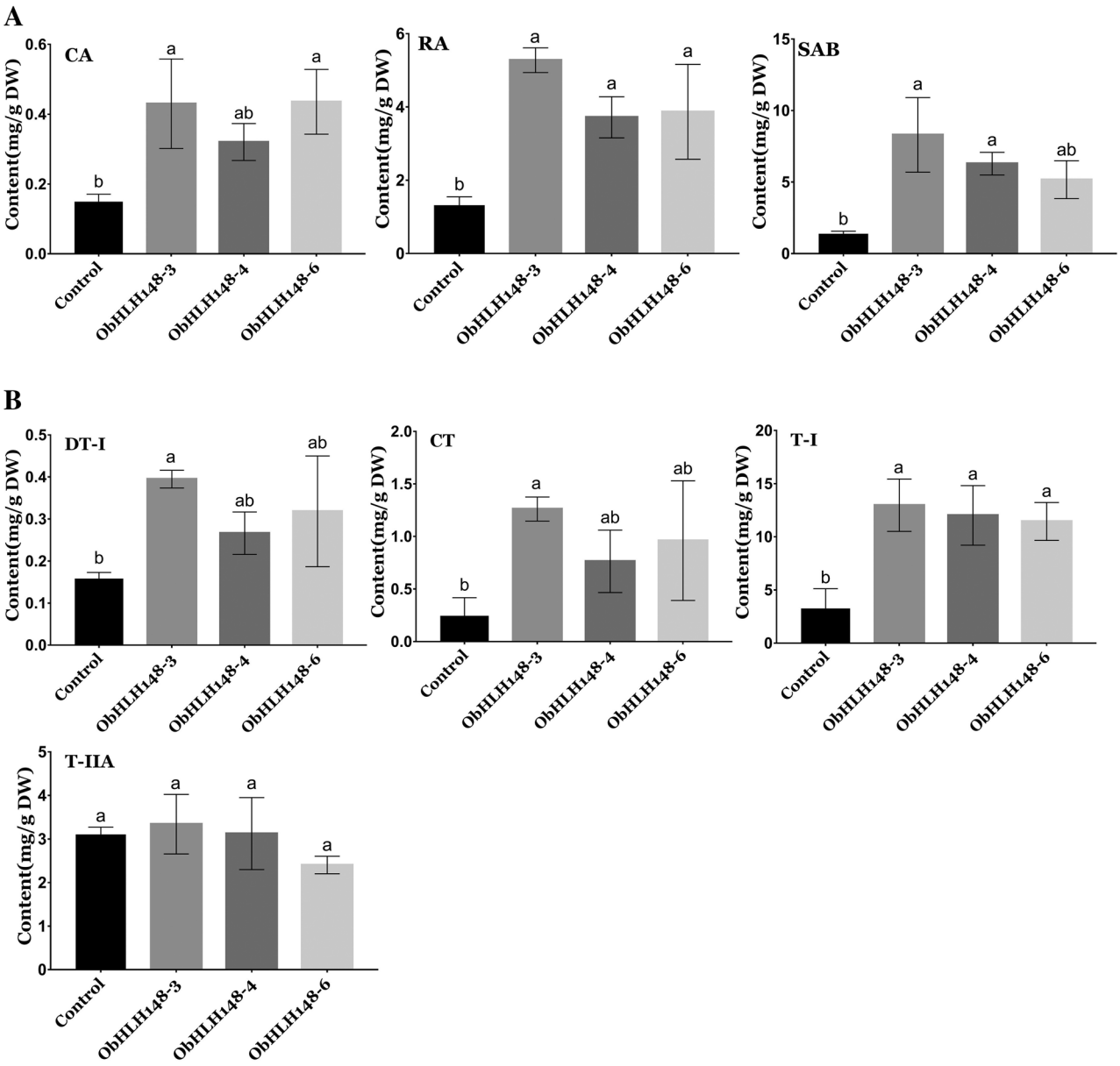

Fig. 6 **a** Content of CA (caffeic acid), RA (rosmarinic acid), SAB (salvianolic acid B) in transgenic and the control hairy roots lines of *S. miltiorrhiza*. **b** Content of T-I (tanshinone I), T-IIA (tanshinone IIA), CT (cryptotanshinone), DT-I (dihydrotanshinone I) in transgenic and the control hairy roots lines of *S. miltiorrhiza*. The vertical bars show the SD values ($n=3$). One-way ANOVA (followed by a Turkey comparison) tested for significant differences among the means (indicated by different letters at $P<0.05$)

pathways of phenolic acids and tanshinones through inducing the enzyme genes and then upregulating the production of phenolic acids and tanshinones.

As bHLH TFs were bound to G-box in gene's promoters and then active/repress the gene expression, the promoter (1500 ~ 2000 bp) region of these pathway genes were analyzed. Except for *RAS* and *CYP76AH1*, G-box like element were present on *PAL1, C4H1, TAT, HPPR, RAS, CYP98A14, DXS2, DXS3, CPS1, CPS5, KSL1* promoter region. We will perform the Y1H and EMSA assay to detect whether SmbHLH148 binds to the promotor of *PAL1, C4H1, TAT, HPPR, RAS, CYP98A14, DXS2, DXS3, CPS1, CPS5, KSL1* and regulate their transcription directly in further study.

bHLH transcription factors often interact with MYB family proteins to form a complex, and then regulate the transcription of target genes (Antje et al. 2011). To investigate whether there has some potential partner of SmbHLH148, expression pattern of all the SmMYB genes in transgenic lines and the control were analyzed (Fig. S3). The results showed that 29 of these *SmMYBs* transcription were

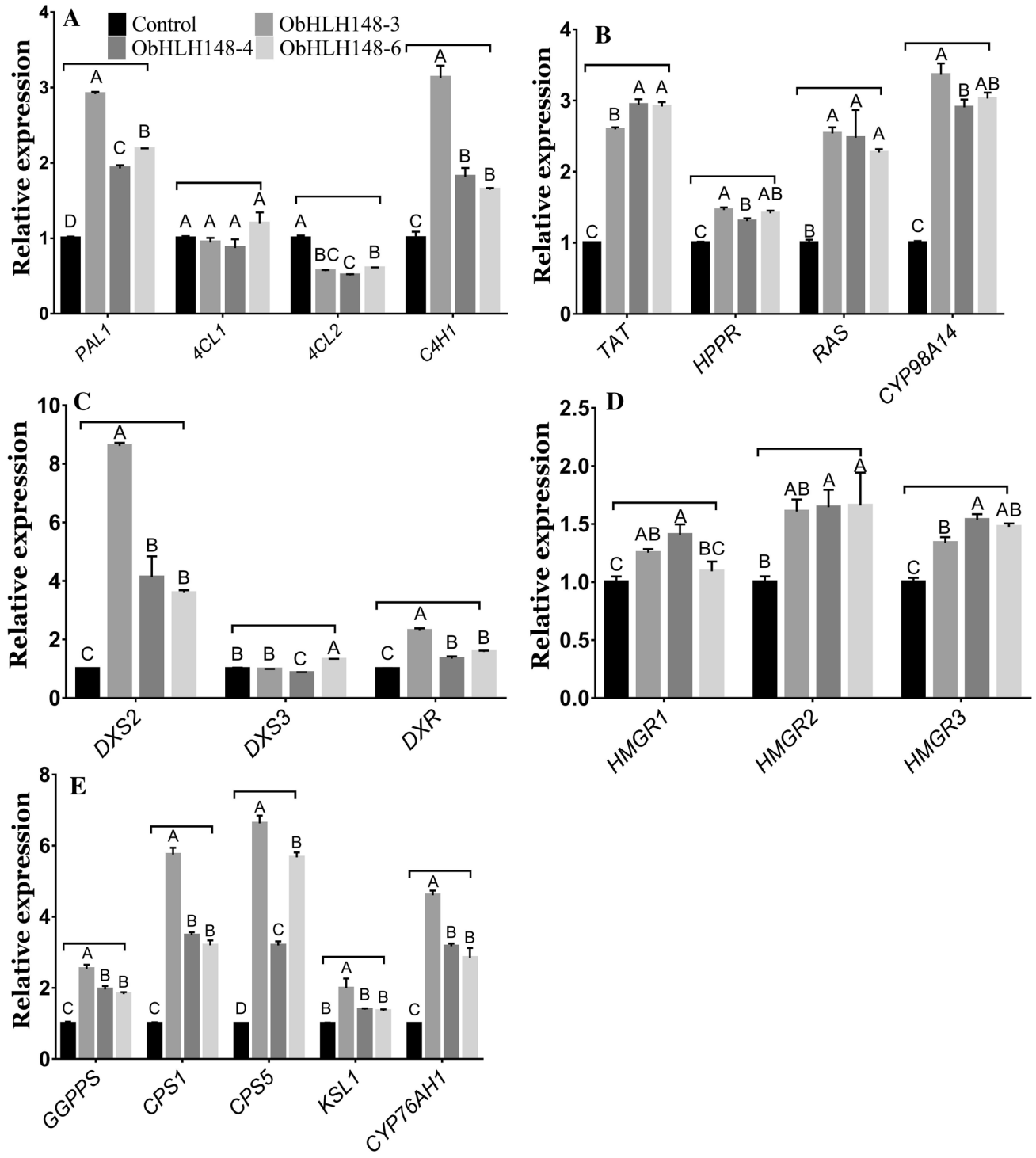

Fig. 7 **a, b** Relative expression level of phenolic acids biosynthesis pathways genes in transgenic hairy roots lines and the control. **c, d, e** Relative expression level of tanshinones biosynthesis pathway genes in transgenic hairy roots lines and the control. The vertical bars show the SD values ($n=3$). One-way ANOVA (followed by a Turkey comparison) tested for significant differences among the means (indicated by different letters at $P<0.01$)

up-regulated in the SmbHLH148 overexpression lines, especially the expression of *SmMYB61, SmMYB79, SmMYB84* and *SmMYB102*. And there were 23 of the *SmMYBs* exhibited relatively low expression in SmbHLH148 overexpression lines. There may be one or more potentially MYB family proteins which SmbHLH148 may interact with.

However, the function mode of SmbHLH148 is by complex with these MYBs or independently still need further study.

Conclusion

A novel bHLH TF named SmbHLH148 was identified and functionally analyzed in *S. miltiorrhiza*. Overexpression of *SmbHLH148* obviously promoted the biosynthesis of both the two groups bioactive ingredients. *SmbHLH148* intensely responds to ABA, whereas moderately induced by MeJA or GA. Transcription of almost all the key genes involved in phenolic acids and tanshinones biosynthesis was enhanced by SmbHLH148. These findings indicated that SmbHLH148 improved phenolic acids and tanshinones biosynthesis by activating the pathway genes transcript. Nevertheless, whether SmbHLH148 regulating the pathway genes by directly binding their promoters or taking roles in ABA or MeJA signaling still need further studies to explore.

Author contribution statement ZL conceived and designed the research. BX, LJL and LL conducted experiments and analyzed the data. BX wrote the manuscript. ZH and DY contributed advice and revised. KY and XZ provided materials and contributed new reagents, advise.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Nos. 81773835 and 81673536), Natural Science Foundation of Zhejiang Provincial (No. LZ16H280001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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