Transcription factor SmWRKY1 positively promote the biosynthesis of tanshinones in *Salvia miltiorrhiza*

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Abstract

Tanshinones, one group of bioactive diterpenes, were widely used in the treatment of cardiovascular diseases. WRKYs play important roles in plant metabolism, but their regulation mechanism in *S. miltiorrhiza* remains elusive. In this study, one WRKY transcription factor *SmWRKY1* was isolated and characterized from *S. miltiorrhiza*. Multiple sequence alignment and phylogenetic tree analysis showed *SmWRKY1* shared high homology with other plant WRKYs such as *CrWRKY1*. *SmWRKY1* were predominantly expressed in leaves and stems, and was responsive to salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO) treatment. Subcellular localization analysis found that *SmWRKY1* was localized in the nucleus. Over-expression of *SmWRKY1* significantly elevated the transcripts of genes involved in MEP pathway especially 1-deoxy-D-xylulose 5-phosphate synthase (*SmDXS*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*SmDXR*), resulted in over 6 folds increase in tanshinones production in transgenic lines (up to 13.731mg/g dry weight (DW)) compared with the control lines. Dual-luciferase (Dual-LUC) assay showed that *SmWRKY1* can positively regulate *SmDXR* expression by binding to its promoter. Our work revealed that *SmWRKY1* participated in the regulation of tanshinones biosynthesis and acted as a positive regulator through activating *SmDXR* in the MEP pathway, thus discloses a new insight to further excavate the regulation mechanism of
tanshinones biosynthesis.

**Keywords:** *Salvia miltiorrhiza*; hairy roots; *SmWRKY1*; MEP pathway; tanshinones; metabolic engineering

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**Introduction**

*Salvia miltiorrhiza* Bunge, belonging to the *Lamiaceae* family, is a famous and prevalent Chinese herbal plant that has been widely used for the treatment of cardiovascular and cerebrovascular diseases (Zhang et al., 2010; Kai et al., 2011). The abietane-type diterpenes in *S. miltiorrhiza* are the liposoluble tanshinones including dihydrotanshinone, tanshinone I, tanshinone IIA and cryptotanshinone, which exert a variety of biological activities such as antioxidant, heart-protection, antibacterial and antitumor (Zhang et al., 2011; Chen et al., 2012; Gong et al., 2012; Xu et al., 2010, 2015). However, serious quality decrease and the low content of tanshinones in cultivated *S. miltiorrhiza* greatly limited the increasing market need (Hao et al. 2015; Zhou et al., 2016a). Therefore, it is important to improve the content of tanshinones by genetic engineering, which relies on deep understanding of the tanshinone biosynthetic pathway to *S. miltiorrhiza* (Liao et al., 2009; Zhou et al., 2016a). Tanshinones derived from the terpenoids metabolism including the mevalonate (MVA)
pathway and the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway, both consisted
of a series of complex enzyme catalytic reactions while operated in separate
subcellular compartments, the MVA pathway localized in the cytosol and the MEP
pathway took place in plastids (Kai et al., 2011; Shi et al., 2016a). Isopentenyl
pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) from the
MEP pathway are the universal C5 precursors of tanshinones, therefore, tanshinone
are generally considered to be mainly derived from the MEP pathway (Ge and Wu,
2005; Yan et al., 2009; Kai et al., 2011; Zhou et al., 2016). Recently, several key genes
including 3-hydroxy-3-methylglutaryl CoA reductase (HMGR),
1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR),
1-deoxy-D-xylulose-5-phosphate synthase (DXS), geranylgeranyl diphosphate
synthase (GGPPS), copalyl diphosphate synthase (CPS), kaurene synthase (KS),
miltiradiene oxidase (CYP76AH1) have been successfully cloned and characterized
from S. miltiorrhiza (Liao et al., 2009; Yan et al., 2009; Kai et al., 2010; Ma et al.,
2012; Shi et al., 2014; Gao et al., 2009; Guo et al, 2013; Zhou et al., 2016a).
Clarification of the above key genes involved in the tanshinones biosynthetic pathway
enabled us to produce elevated concentration of tanshinones in S. miltiorrhiza through
genetic engineering by manipulating one or several regulation points in either MVA or
MEP pathway (Kai et al., 2011; Shi et al., 2014; Shi et al., 2016a). Co-expression of
SmHMGR and SmGGPPS increased tanshinone production significantly in a
transgenic S. miltiorrhiza hairy root line HG9 (Kai et al., 2011). In addition,
introduction of SmHMGR and SmDXR into S. miltiorrhiza hairy roots enhanced the
content of tanshinones apparently (Shi et al., 2014). Simultaneous introduction of
SmGGPPS and SmDXSII into S. miltiorrhiza hairy root significantly improved the
production of tanshinones, besides, their expression in Arabidopsis thaliana plants
increased production of carotenoids, gibberellins and chlorophyll in contrast to the
non-transgenic lines (Shi et al., 2016a).

Apart from manipulation of pivotal catalytic steps in the biosynthetic process of
tanshinones, regulation of transcription factors such as MYB and bHLH transcription factor family was also considered as a feasible strategy to mine the biosynthesis mechanism of tanshinones (Zhou et al., 2016; Zhang et al., 2017). Heterologous expression of maize transcription factor C1 in *S. miltiorrhiza* hairy roots elevated the accumulation of tanshinones through direct interaction of C1 with its recognition sequences of pathway genes, especially mevalonate-5-diphosphate-decarboxylase (*SmMDC*) and 5-phosphomevalonate kinase (*SmPMK*) to upregulate their expression levels (Zhao et al., 2015). RNA interference (RNAi) of *SmMYC2a/b* affected multiple genes in tanshinone biosynthetic pathway and led to a reduction of tanshinones contents, implying that *SmMYC2a/b* may be a positive regulator of tanshinones accumulation (Zhou et al., 2016b). Overexpression of a *S. miltiorrhiza* R2R3-MYB gene *SmMYB9b* resulted in a 2.2-fold enhancement of tanshinones accumulation in danshen hairy roots over the control (Zhang et al., 2017). However, less is known about WRKY transcription factors and their regulation mechanism in *S. miltiorrhiza* (Li et al., 2015).

Salicylic acid (SA) is a kind of plant hormone signal in plant metabolism, which is also reported that it could induce the accumulation of tanshinone as reported before (Hao et al., 2015), but its regulation mechanism is not clear yet. WRKY transcription factors form one of the largest gene families unique to plants, which are involved in plant secondary metabolism (Suttipanta et al., 2011). The first WRKY gene named *SPF1*, was identified from sweet potato (Ishiguro and Nakamura, 1994). Subsequently, much attention has been paid to identify and analyze WRKY genes from different model and crop plants, for instance *Arabidopsis* (Eulgem et al., 2000; Kalde et al., 2003; Wang et al., 2011), soybean (*Glycine max*) (Yin et al., 2013), tobacco (Yoda et al., 2002), rice (*Oryza sativa*) (Wu et al., 2005) and so on.

Meanwhile, WRKY has been isolated from some traditional herbal plants including *Artemisia annua*, *Coptis japonica* and *Catharanthus roseus* (Jiang et al., 2015; Chen et al., 2017; Kato et al., 2007; Suttipanta et al., 2011). The significant feature of
WRKY transcription factor is their WRKY domain which is approximately 60-amino acid long with the highly conserved amino acid sequence WRKYGQK located at the N-terminal and a non-typical zinc-finger-like motif C2HC (C–X7–C–X23–H–X1–C) or C2H2(C–X4–5–C–X22–23–H–X1–H) at the C-terminus (Xu et al., 2004; Lu et al., 2015). WRKY proteins can bind to the W-box cis-elements (T)TGAC(C/T) in the promoter region of some defense-related genes (Xu et al., 2004; Rushton et al., 2010; Liu et al., 2016). WRKY transcription factors can be separated into three sub-groups in accordance with the number of specific WRKY domains and zinc-finger-like motifs, Group I contains two WRKY domains and C2H2 motif, Groups II has one WRKY domain and C2H2 motif and Group III possesses one WRKY domain and C2HC motif (Eulgem et al., 2000; Rushton et al., 2010). WRKYs have shown many different functions on multiple physiology activities including stress defense, trichome development and secondary metabolism (Jiang et al., 2016). For example, *Gossypium arboretum* WRKY1 (GaWRKY1) was found to participate in regulation of sesquiterpene biosynthesis in cotton by regulate the target gene (+)-delta-cadinene synthase (CAD1) (Xu et al., 2004). *C. roseus* WRKY1 (CrWRKY1) bound to the W-box elements of the tryptophan decarboxylase (TDC) promoter involved in terpenoid indole alkaloid (TIA) biosynthetic pathway and accumulated up to 3-fold higher levels of serpentine compared with control hairy roots (Suttipanta et al., 2011). The WRKY transcription factor *GLANDULAR TRICHOME-SPECIFIC WRKY 1* (AaGSW1) positively regulated the expression of AaCYP71AV1 and AaORA by conjunction to the W-box motifs in their promoters (Chen et al., 2017). *Glycine max* WRKY27 responsive to various abiotic stresses interacted with GmMYB174, and then cooperatively inhibited GmNAC29 expression, facilitating stress-tolerance of drought and cold in soybean (Wang et al., 2015). A WRKY transcription factor from *W. somnifera* bound to the W-box region in the promoters of squalene synthase and squalene epoxidase, regulating the accumulation of triterpenoids in *W. somnifera* including phytosterols and withanolides (Singh et al., 2017). However, functional
WRKYs related to secondary metabolism of tanshinones or salvianolic acids in *S. miltiorrhiza* have not been reported.

In this study, a WRKY transcription factor has been isolated from *S. miltiorrhiza* (named as *SmWRKY1*) and functionally characterized. Phylogenetic analysis showed that it shared high homology with AtWRKY70, CrWRKY1 and GaWRKY1. Multiple sequence alignment revealed that the nucleus-localized *SmWRKY1* contained one WRKY domain, with conserved amino acid sequence WRKYGQK and a C2HC type zinc-finger-like motif, therefore it can be classified into group III WRKY transcription factors. Introduction of *SmWRKY1* into *S. miltiorrhiza* hairy roots improved the transcripts of *SmDXS* and *SmDXR* involved in MEP pathway, resulting in higher level of tanshinones in transgenic lines compared with the control lines (2.175mg/g DW). The highest content of tanshinones was detected in *SmWRKY1-3* at 13.731mg/g DW, which was 5.3 folds higher than the control. Dual-LUC assay revealed that *SmWRKY1* activated the expression of *SmDXR* by binding to the promotor region containing one w-box in vivo. Taken together, our work revealed that *SmWRKY1* positively elevated the accumulation of tanshinones, which provides a new insight to further excavate the regulation mechanism of tanshinones biosynthesis.

### Materials and Methods

**Plant samples and reagents**

*S. miltiorrhiza* seedlings used for *Agrobacterium*-mediated transformation were cultivated in Murashige and Skoog (MS) medium (pH5.8) containing 3% sugar and 0.8% agar in the greenhouse, growth conditions were as follows: 16 h: 8 h, light: dark cycle under 25°C with 60% relative air humidity as reported before (Kai et al., 2011; Shi et al., 2014, 2016a). Seeds of *N. benthamiana* were sown and cultivated in the pots supplemented with soil matrix for 4-5 weeks for infiltration (Zhou et al., 2016a).
All strains (Escherichia coli DH5α, Agrobacterium C58C1, GV3101 and ASE) and plasmid vectors (pCAMBIA2300, pMON530) used in this paper were preserved in our laboratory. The intermediate cloning vector pMD-18T and reverse transcriptase M-MLV were purchased from TaKaRa Biotechnology Co., Ltd. Primers-synthesizing and DNA sequencing were performed by Shanghai Sangon Biotechnological Company, China. RNA extraction kit and qRT-PCR kit were purchased from Tiangen Company. Standards of cryptotanshinone, tanshinone I, tanshinone IIA, dihydrotanshinone used for HPLC analysis were purchased from Aladdin, China. MJ, SA and SNP used for elicitation treatments were purchased from Sigma-Aldrich, Sinopharm Chemical Reagent Co., Ltd, respectively.

**Elicitor preparation**

For methyl jasmonate (MeJA) induction, MeJA was dissolved in 5% ethanol, and then dissolved into distilled water to a storage concentration of 50 mM. A final working concentration of 100 µM MeJA was employed for elicitation assay, and equivalent volume of sterilized water was used as the mock treatment (Kai et al., 2012). For salicylic acid (SA) treatment, SA was dissolved in sterile water to a storage concentration of 50 mM, and then added to hairy roots cultures to the final concentration of 100 µM (Hao et al., 2015). For NO elicitation, first a concentration of 100 mM SNP solution was obtained, and then applied to cultures to 100 µM. All the above-mentioned solutions were sterilized through 0.22μm filters (Pall Corporation, USA). And solvent of the equivalent volume was added into the control group.

**Identification and cloning of SmWRKY1**

A local transcription database of S. miltiorrhiza built up as reported previously (Shi et al., 2016b) was used for this research. One partial WRKY in high homology with other plants WRKYs while lack of 3’-terminal was chosen for further study. Gene-specific
forward primer SmWRKY1-F605 was designed to amplify the 3’ end of SmWRKY1 as well as the reverse primer AUAP by rapid amplification of cDNA ends (RACE) (Liao et al., 2009; Kai et al., 2010; Zhang et al., 2011). 5’-sequence and 3’-terminal products was aligned and assembled to obtain the full-length cDNA sequence of the putative SmWRKY1 gene. Primer pairs SmWRKY1-KF and SmWRKY1-KR were synthesized for amplification of the full ORF of SmWRKY1 according to the procedure as described below: initial denaturation at 94 °C for 10 min, 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. All primers used for identification of SmWRKY1 were listed in Supplemental Table 1.

**Bioinformatics analysis of SmWRKY1**

Biological characteristics of SmWRKY1 were further analyzed by a series of tools. Nucleotide blast, protein blast and ORF Finder were used to analyze nucleotide sequence and complete open reading frame. MEGA 6 was applied to construct a phylogenetic tree by the neighbor-joining (NJ) method and 1000 replications were performed for bootstrap values. Multiple sequences alignment between SmWRKY1 and other plant WRKYs were carried out using Clustal X with default parameters (Shi et al., 2016b; Zhou et al., 2016a).

**Expression pattern of SmWRKY1 in different tissues and under various elicitors treatments**

Different tissues including taproot, stem, leaf, flower and seed were gathered from two-year-old *S. miltiorrhiza* plants in mature. Elicitor treatments were conducted on *S. miltiorrhiza* hairy roots sub-cultured for 60 days infected with *Agrobacterium C58C1*. Hairy roots were harvested at selected time points (0h, 0.5h, 1h, 2h and 4h) after MJ treatment. And for SA and NO induction, hairy roots were collected at 0h, 3h, 4h, 6h, 9h, 12h after treatment. All the treated samples were immediately frozen in liquid
nitrogen and stored for analyzing the expression profiles of *SmWRKY1*.

**Subcellular localization of *SmWRKY1***

To analyze the subcellular localization of *SmWRKY1*, PCR products of *SmWRKY1* ORF with *Bgl*II and *Kpn*I restriction sites were digested with *Bgl*II and *Kpn*I and cloned into the vector *pMON530* to generate the vector *pMON530-SmWRKY1-GFP*. The constructed expression vector was transferred into *Agrobacterium* strain ASE and injected into forty-day-old tobacco leaves. GFP fluorescence was observed after 48h cultivation using the confocal microscope (Carl Zeiss) (Shi et al., 2016b; Zhou et al., 2016a).

**Generation of transgenic *SmWRKY1* hairy roots**

The full-length coding sequence of *SmWRKY1* with restriction sites *Spe* I and *Bst*EII was cloned and inserted into modified *pCAMBIA2300sm* vector (replace the small fragment digested by *Eco* I and *Hind* III with the corresponding GFP-GUSA gene expression cassette from *pCAMBIA1304*) under the control of the CaMV 35S promoter to generate *pCAMBIA2300sm-SmWRKY1* as described before (Shi et al., 2016b). *A. rhizogenes* strain C58C1 containing *pCAMBIA2300sm-SmWRKY1* was used to infect the aseptic explants and the empty *pCAMBIA2300sm* was regarded as the control. The transformation procedure was the same as our previous study (Kai et al., 2011; Shi et al., 2014, 2016a, 2016b; Zhou et al., 2016a). Hairy roots in good state were sub-cultured and primer pairs *CaMV35S*-F23 and *SmWRKY1*-QR were used to identify the positive colony by polymerase chain reaction (PCR) analysis, meanwhile *rolB* gene in C58C1 was detected. Genomic DNA was isolated from individual hairy root sample by the cetyltrimethyl ammonium bromide method as previously reported (Zhou et al., 2016a, c). Identified positive-colonies were segmented approximate 4 cm long for shake-flask culture in 100 mL 1/2MS medium and cultured at 25°C on an orbital shaker shaking at the speed of 100 rpm in darkness (Shi et al., 2016a, 2016b). Primers sequences were listed in Supplemental Table 1.
Total RNA isolation and relative expression analysis via qRT-PCR

Expression profiles of *SmWRKY1* and several key enzyme genes involved in tanshinones biosynthetic pathway were investigated by real-time quantitative PCR analysis (qRT-PCR). Total RNA was extracted from different tissues with the RNA prep pure plant kit as described before (Shi et al., 2016b). Total RNA served as the template for reverse transcription (RT) reaction, the reaction conditions were according to our previous study (Shi et al., 2016b). Gene-specific primers (listed in Supplemental Table 1) for qRT-PCR were designed and analyzed the relative expression level compared with the internal reference gene *SmActin* using the relative quantitative analysis method (2^−ΔΔCT). Amplifications were performed according to the manufacturer’s instructions: one cycle of denaturation at 95 °C for 10 min, then 40 cycles of 15 s denaturation at 95 °C, 30 s annealing at 60 °C and 30 s extension at 72 °C.

Dual-Luciferase (Dual-LUC) assay

For the dual-luciferase (Dual-LUC) assay, the promoters of *SmDXR* and *SmDXS2* with *KpnI* and *XhoI* restriction sites were cloned into pGREEN 0800 to drive the luciferase reporters, respectively. And the complete ORF of *SmWRKY1* was inserted into the *pCAMBIA2300* vector as effector. The *pCAMBIA2300*-SmWRKY1 and *pCAMBIA2300* empty plasmid were transferred into *Agrobacterium tumefaciens* strain GV3101 individually. The *pGREEN-pSmDXR*, *pGREEN-pSmDXS2* was each co-transformed with the helper plasmid pSoup19 into GV3101, and the assay was conducted as described before (Zhang et al., 2015). The *pCAMBIA2300* empty plasmid was used as a negative control. The 35S promoter-driven Renilla was taken as an internal control. Each sample were measured for three biological times. The reporter strain with effector strain was mixed with ratio of one-to-one to inject the tobacco leaves. After two days' injection, the samples were collected for dual-LUC assay by reaction reagents according to the manufacturer (Promega).
**Tanshinones analysis**

The 60-day-old hairy roots were dried at 50 °C to constant weight in an oven. Approximate 200 mg dried hairy roots were ground into powder and immersed in 16 mL methanol/dichloromethane (3:1, v/v) for tanshinones extraction. Tanshinones extraction was carried as reported before (Hao et al., 2015). HPLC analysis was performed on Agilent 1260 apparatus equipped with a Waters reversed-phase C18 symmetry column, and the detection conditions were performed following the methods described previously (Shi et al., 2016b).

**Results**

**Isolation and molecular cloning of SmWRKY1**

WRKY transcription factor is a large family in plants which has been proven to be involved in the regulation of many physiological processes in plants including secondary metabolism (Xu et al., 2004; Suttipanta et al., 2011). By searching our local transcriptome database, a WKRY fragment with 5' untranslated region (UTR) but lack of partial of 3' terminal sequence was chosen for further research because it showed high homology with GaWRKY1 and CrWRKY1 as well as Arabidopsis thaliana WKRY70. 3' RACE technology was used to obtain a 432 bp sequence of 3' end of the fragment. After sequence assembly, the full-length gene was cloned and designated it as SmWRKY1. SmWRKY1 sequence consists of 17 bp 5'UTR, a complete 789 bp open reading frame which encodes 262 amino acids, along with 238 bp 3' UTR.

**Bioinformatics analysis of SmWRKY1**

To further figure out the biological characteristics and phylogenetic relationship of SmWRKY1, a series of bioinformatics analysis were performed. Multiple alignment of SmWRKY1 with related WRKY proteins from other plant species revealed that SmWRKY1, AaWRKY1 and CrWRKY1 all contained a conserved WRKY domain (WRKYGQK) and a special zinc finger like motif in its C-terminal which falls into
the group III of WRKY family (Fig. 1A) and indicated that they might have similar function. Then alignment of SmWRKY1 and other plant WRKYs was performed at amino acid level and a neighbor-joining tree was constructed, as shown in (Fig. 1B). The results revealed that SmWRKY1 shared 62%, 49%, 37%, 29% identities with EgWRKY70, NtWRKY70, CrWRKY1 and AaWRKY1, respectively.

**Tissue and induction expression profiles of SmWRKY1**

To investigate the tissue expression pattern of SmWRKY1, roots, stems, leaves, flowers and seeds from two-year-old *S. miltiorrhiza* plants were analyzed. SmWRKY1 showed significant expression in leaves and stems and low expression in flower and root, its transcript was barely detected in seeds (Fig. 2A). This result indicated that SmWRKY1 was not a tissue-constitutive expression gene.

To study whether SmWRKY1 could respond to exogenous hormone treatment, 60-day-old *S. miltiorrhiza* hairy roots were treated with MeJA for different time points while the 0 hr point was used as control and the expression was detected by qRT-PCR. The result indicated that SmWRKY1 expression was induced by exogenous MeJA (Fig. 2B), the expression level reached peak at 0.5h after treatment, arising approximate 3-fold compared with control). Then, the transcript level of SmWRKY1 declined rapidly in two hours. Meanwhile, the hairy roots were also treated with SA and NO. Both SA and NO could induce the expression of SmWRKY1, which reached the maximum level at 3h and gradually decreased till 12h after treatment (Fig. 2C, D). In summary, SmWRKY1 could be induced by MeJA, SA and NO.

**Subcellular localization of SmWRKY1**

To experimentally confirm the subcellular localization of SmWRKY1, SmWRKY1 was cloned into the *pMON530* vector to fuse with green fluorescent protein (GFP) reporter gene to generate vector *pMON530-SmWRKY1-GFP*. Then, the constructed vector and the *pMON530* (used as the control) was transformed into ASE strain and expressed in
tobacco leaves, respectively. In the leaves of control vector transformed plant, the fluorescence of GFP was detected in the cytoplasm and nucleus (Fig. 3). On the contrast, the fluorescent signal of SmWRKY1-fused GFP was only examined in nucleus. The expression pattern was consistent with the character of SmWRKY1 as a transcription factor.

Acquisition of SmWRKY1 transgenic hairy roots

To further investigate the function of SmWRKY1 in S. miltiorrhiza, we inserted SmWRKY1 into a modified pCAMBIA2300\textsuperscript{sm} vector. Then, the recombinant overexpression vector pCAMBIA2300\textsuperscript{sm}-SmWRKY1 was introduced into A.rhizogenes strain C58C1 and used to infect S. miltiorrhiza explants and the empty vector pCAMBIA2300\textsuperscript{sm} was used as control. After 2-3 weeks the fresh hairy roots differentiated from the stem and leaf explant as shown in Fig. 4. The positive lines carrying SmWRKY1 gene were verified by PCR. The positive rate was 20.5% among the 39 samples (Fig. 5). qRT-PCR analysis of the expression of SmWRKY1 in over-expression lines found that SmWRKY1 expressed 20- to 48-fold higher than the empty vector control transformed lines (Fig. 6A). The three high expression lines including 1, 2 and 32 (designated as 3) were chosen for further analysis.

SmDXS and SmDXR involved in MEP pathway were up-regulated by SmWRKY1

To study whether SmWRKY1 participated in the regulation of tanshinone biosynthesis, transcript levels of several genes related to tanshinones biosynthesis in SmWRKY1 transgenic hairy root were analyzed by qRT-PCR. Several tanshione biosynthesis pathway genes were up-regulated in the SmWRKY1-overexpressing hairy roots (Fig. 6B), the most striking ones were SmDXS2 and SmDXR gene, which increased 4-6 folds and 4-10 folds compared with the control, respectively. Though the expression of SmIPP1, SmGGPPS, SmCPS, SmKSL and SmCYP76AH1 was a little lower than SmDXS and SmDXR, their expression in over-expression lines was 2-4 folds higher.
than the control. In contrast, the expression of all these seven tanshinones biosynthesis pathway genes were significantly decreased in the knock-down lines. All these results suggested that SmWRKY1 may be a positive regulator in tanshinones biosynthesis.

**SmWRKY1 activates the transcription of SmDXR in vivo**

Expression profiles showed that SmWRKY1 significantly promote the expression of SmDXR and SmDXS2 in charge of pivotal catalytic steps of tanshinone accumulation. By analyzing the sequence of SmDXR and SmDXS2 promoter, we found a W-box in the promoter of SmDXR (Fig. 7A). Than dual luciferase (dual-LUC) method was employed to verify whether SmWRKY1 protein activates the transcription of SmDXR and SmDXS2 or not. The results showed that SmWRKY1 elevated the expression of SmDXR by 6.08-fold (Fig. 7B) while endowed inconspicuous change.

**Accumulation of tanshinone was obviously affected by SmWRKY1**

Based on the quantitative data, we wanted to further evaluate whether the expression of SmWRKY1 in transgenic hairy roots affect the content of tanshinone. Three overexpression lines and two knock-down line were used to examine four monomers of tanshinone including cryptotanshinone, dihydrotanshinone I, tanshinone I, tanshinone IIA in hairy roots by HPLC. The results showed that the content of cryptotanshinone, dihydrotanshinone I, tanshinone I were significantly up-regulated and the total tanshinone had risen to 9.443-13.731mg/g DW in over expression lines. Among them pCAMBIA2300^sm^-SmWRKY1-3 lines accumulated the highest content of total tanshinone, which was 6.31 folds higher than control (Fig 8). These results further confirmed the positive role of SmWRKY1 in the regulation of tanshinone biosynthesis.

**Discussion**
WRKY transcription factors are one of the largest gene families specific to plants which have been studied for decades. The conserved domain WRKYGQK and a zinc finger motif which consists of 60 amino acids are considered as the general character of WRKY TFs which also can be regarded as the criterion for subgrouping (Eulgem et al., 2000; Xie et al., 2005; Zhang and Wang, 2005). SPF1, ABF1.2, PcWRKY1.2.3 and ZAPI are the first WRKY cDNAs isolated from sweet potato (Ipomoea batatas), wild oat (Avena fatua), parsley (Petroselinum crispum) and Arabidopsis, respectively (Ishiguro et al., 1994; Rushton et al., 1996; de Pater, S. et al. 1996). Up to now, 74 and 109 WRKYs members have been found in Arabidopsis and Oryza sativa respectively (Ujjal et al., 2016). Previous studies have proved that WRKY TFs could directly bind to the W-box of related genes from different signal pathways and played its regulatory role in stress tolerance in plants (Eulgem et al., 2000). For instance, SpWRKY1 has been testified to promote resistance to Phytophthora nicotianae and tolerance to salt and drought stress in transgenic tobacco (Li et al., 2015). GhWRKY25 from cotton, a member of group I, conferred transgenic Nicotiana benthamiana differential tolerance to abiotic and biotic stresses (Liu et al., 2016). In recent years, the role of WRKY TFs in the regulation of secondary metabolism in plants has gained attentions, and some progress has been made in this field, for example, the involvement of Artemisia annua WRKY1 (AaWRKY1) transcription factor can elevate the production of artemisinin by targeting the Amorpha-4,11-diene synthase (ADS) gene of Artemisia annua (Ma et al., 2009; Jiang et al., 2016). A jasmonate- and salicin-inducible WRKY transcription factor from Withania somnifera named as WsWRKY1 could bind to W-box sequences in promoters of squalene synthase and squalene epoxidase genes in W. somnifera genes regarding triterpenoid biosynthesis such as phytosterol and withanolides (Singh et al., 2017). The WRKY transcription factor GLANDULAR TRICHOME-SPECIFIC WRKY 1 (AaGSW1) positively regulated the expression of AaCYP71AV1 and AaORA by conjunction to the W-box motifs in their promoters (Chen et al., 2017). However, lack of research on the...
function of WRKY TFs in *S. miltiorrhiza* especially in the regulation of tanshinone biosynthesis were reported. *S. miltiorrhiza*, a traditional Chinese herbal medicine, has been used for thousands of years. Previous studies have proved that as a major medicinal active ingredient in *S. miltiorrhiza*, tanshinones could be used for the treatment of cardiovascular and cerebrovascular diseases in China (Chen et al., 2012). However, traditional *S. miltiorrhiza* production cannot meet the growing clinical needs due to its slow growth, low tanshinone content and scarcity of wild resources (Zhou et al., 2016a). Thus, genetic engineering has become an effective and important way to increase the accumulation of active ingredients in *S. miltiorrhiza*. Overexpression of *SmDXS* in transgenic hairy root lines can significantly enhance the production of tanshinones (Zhou et al., 2016a). Meanwhile *SmDXR* was also an important enzyme gene in tanshinone biosynthetic pathway whose overexpression could significantly improve the production of tanshinones in hairy root lines (Shi et al., 2014). In our study, a new WRKY transcription factor was successfully cloned from *S. miltiorrhiza* with high homology with *CrWRKY1* and *GaWRKY1*. qRT-PCR analysis showed that over-expression of *SmWRKY1* can promote the transcripts level of *SmDXR* and *SmDXS2* to the greatest extent in comparison to other genes involved in tanshinone biosynthetic pathway such as *SmIPPI*, *SmGGPPS*, *SmCPS*, *SmKSL* and *SmCYP76AH1*. Otherwise, dual-Luciferase (Dual-LUC) assay showed that *SmWRKY1* can positively regulate *SmDXR* expression by directly binding to the promoter region containing one W-box. HPLC analysis revealed that introduction of *SmWRKY1* in transgenic *S. miltiorrhiza* hairy roots can increase the tanshinones production up to 13.731mg/g dry weight (DW) which is over 6 folds as that in non-transgenic lines. Therefore, it is an effective strategy to regulate the tanshinone production in *S. miltiorrhiza* by introduction of related transcription factors.

To our knowledge, the defense mechanisms in plants are complicated and are mainly considered to be regulated by SA and MJ signaling network (Tsuda et al., 2009). SA
plays a vital role in plant defense against pathogens and pathogen invasion obviously.
triggers its accumulation in plants (Qiu et al., 2009). MeJA is widely used as an elicitor to investigate the biosynthetic pathway of active compounds and the underlying regulatory mechanisms (Gundlach et al., 1992). It has been proved to be defensive to environmental stresses such as wounding, pathogen and pest attack, ozone exposure, ultraviolet radiation and salt stress as a regulator (Ma et al., 2006; Wang et al., 2011). While in *S. miltiorrhiza*, exogenous MeJA treatment can promote the accumulation of tanshinone (Gu et al., 2012; Kai et al., 2012; Hao et al., 2015). In our study, we noticed that *SmWRKY1* can be induced by exogenous MeJA treatment, reaching a maximal level at 0.5 h after MeJA treatment., which is consistent with the previous reports that MeJA treatment could increase tanshione production (Hao et al., 2015; Zhou et al., 2017). Recent studies showed that *Brassica napus* WRKY33 (BnWRKY33), a *S. sclerotiorum*-responsive gene, could positively regulate resistance to *S. sclerotiorum* by enhancing the expression of genes involved in camalexin synthesis and genes regulated by salicylic acid (SA) and jasmonic acid (JA) (Liu et al., 2017). JcWRKY a salicylic acid-inducible TF was able to work in co-ordination with SA signaling to orchestrate the different biochemical and molecular pathways to manoeuvre salt stress tolerance of the transgenic tabacco plants (Agarwal et al., 2016). Expression profiles revealed that the *SmWRKY1* was responsive to both SA and MJ, which implied that *SmWRKY1* may participate in the process of stress regulation such as the defense against pathogen, however need to be examined furthermore.

In conclusion, our work revealed a new transcription factor *SmWRKY1* which is involved in the regulation of tanshinone biosynthesis and promote the accumulation of tanshinone in transgenic hairy root lines by targeting *SmDXR* involved in the MEP pathway. Our study may provide a new insight by genetic engineering strategy with functional transcription factors to improve the yield of target compounds in *S. miltiorrhiza*.
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Figure legends

Figure 1 (A) Multiple alignment of SmWRKY1 with related WRKY proteins from other plant species. Black boxes indicate identical residues; grey boxes indicate identical residues for at least three of the sequences. (B) Phylogenetic tree analysis of SmWRKY1 and WRKY TFs from Arabidopsis thaliana, Artemisia annua, Catharanthus roseus, Nicotiana tabacum, etc. Phylogenetic tree was constructed on MEGA6.0 by using neighbor-joining method and the bootstrap values were obtained for 1000 replications.

Figure 2 (A) Expression pattern of SmWRKY1 in different tissues. Each tissue was obtained from several individual two-year-old S. miltiorrhiza plants in nature. Transcript abundance of SmWRKY1 is normalized to actin by the method of 2^{-ΔΔCt}. (B) The expression level of SmWRKY1 after MeJA treatment for different time points by qRT-PCR analysis respectively. (C) The expression level of SmWRKY1 after SA treatment for different time points by qRT-PCR analysis respectively. (D) The expression level of SmWRKY1 after NO treatment for selected points by qRT-PCR analysis respectively.

Figure 3 Subcellular localization of SmWRKY1. (A-D) The free GFP expressed in N. benthamiana leaves. (E-H) SmWRKY1:: GFP expressed in N. benthamiana leaves.

Figure 4 Generation of transgenic hairy root of S. miltiorrhiza. (A) S. miltiorrhiza
explants on ½MS medium; (B) The growing hairy root on the infected *S. miltiorrhiza*

exploats. (C) Monocline of hairy root. (D) Hairy roots culture in 1/2MS medium.

**Figure 5** (A) Identification of positive transgenic hairy root lines by PCR. (*GusA*-F and *GusA*-R were used to identify empty vector *pCAMBIA2300* transformed lines (B) Primers *CaMV35S*-F23 and *SmWRKY1*-QR were used to identify the positive colony of *SmWRKY1* overexpression transgenic lines).

**Figure 6** Transcript levels of *SmWRKY1* and genes related to tanshinones biosynthesis in *SmWRKY1* transgenic hairy roots. Expression of *SmWRKY1* were analyzed by qRT-PCR.

**Figure 7** The SmDXR promoter was fused to the luciferase (LUC) reporter and the promoter activity was determined by a transient dual-LUC assay in tobacco. The value of LUC activity/ Renilla (REN) luciferase was regarded as the activating activity. Error bars indicate SD (n = 3). Student’s t-test: *, P < 0.05; **, P < 0.01

**Figure 8** The production of tanshinone in *SmWRKY1* transgenic hairy roots compared with control detected by HPLC.
