



Tanshinone production could be increased by the expression of *SmWRKY2* in *Salvia miltiorrhiza* hairy roots

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ABSTRACT

Tanshinones are the main bioactive diterpenes in *Salvia miltiorrhiza* Bunge, are widely used for treating cardiovascular and cerebrovascular diseases. However, the biosynthetic mechanisms of these compounds have not yet been fully explained. In this study, a transcription factor named *SmWRKY2* was isolated and functionally characterized. Multiple sequence analysis indicated it was classified into subgroup I of the WRKY family. Expression pattern showed that *SmWRKY2* was mainly expressed in the stem and leaf and was inducible by methyl jasmonate (MeJA) treatment. Subcellular localization showed that *SmWRKY2* was localized in the nucleus. Overexpression of *SmWRKY2* in *S. miltiorrhiza* hairy roots significantly increased the expression of *SmDXS2* and *SmCPS*, resulting in increased accumulation of tanshinones and the highest total tanshinone content was detected in *OE-SmWRKY2-1* line, which was 1.83 times of the control. Meanwhile, tanshinone production was slightly reduced in the *antisense-SmWRKY2* line. Dual-Luciferase assay showed that *SmWRKY2* can positively regulate *SmDXS2* and *SmCPS* expression. However, Y1H and EMSA experiments indicate that *SmWRKY2* only binds to the W-box of the *SmCPS* promoter. Our study shows that *SmWRKY2* is a positive regulator of tanshinone biosynthesis by mainly activating *SmCPS*. This study thus sheds new light on the regulatory role of *SmWRKY2* in tanshinone biosynthesis.

1. Introduction

Cardiovascular and cerebrovascular diseases are one of the most serious health problems, threatening people's lives all around the world [1]. *S. miltiorrhiza* (also named Danshen), a well-known and valuable traditional Chinese medicine, has been proven to be effective in the treatment of cardiovascular diseases, and this plant has also been developed into various preparations such as the famous Compound Danshen Dripping Pills, which are now widely used in many countries [2–5]. There are mainly two categories of bioactive compounds in Danshen, namely tanshinones and phenolic acids [3,4,6,7]. As one kind of diterpenoids, tanshinones including dihydrotanshinone (DH-TI), tanshinone IIA (T-IIA), cryptotanshinone (CT) and tanshinone I (T-I) are

derived from the universal five-carbon precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) that is synthesized in two independent pathways, the 2-C-methyl-Derythritol 4-phosphate (MEP) pathway that has a plastidial location and the mevalonate (MVA) pathway in the cytosol (Fig. S1) [3,8–11]. Recently, multiple rate-limiting genes involved in tanshinone biosynthesis have been isolated and identified from *S. miltiorrhiza*, such as those encoding for 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), 1-Deoxy-D-xylulose 5-phosphate synthase (DXS2), 1-deoxy-D-5-phosphate reductoisomerase (DXR), geranylgeranyl diphosphate synthase (GGPPS), copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), miltiradiene oxidase (CYP76AH1), to name a few [3,6,12–14]. Manipulating key enzyme genes by genetic engineering individually or

Abbreviations: *S. miltiorrhiza*, *Salvia miltiorrhiza*; T-I, tanshinone I; T-IIA, tanshinone IIA; CT, cryptotanshinone; DH-TI, dihydrotanshinone; TT, total tanshinones; MEP, 2-C-methyl-Derythritol 4-phosphate; MVA, mevalonate; GGPPS, geranylgeranyl diphosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS2, 1-deoxy-D-xylulose 5-phosphate synthase; CPS, copalyl diphosphate synthase; KSL, kaurene synthase-like; CYP76AH1, miltiradiene oxidase CYP76AH1; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; MJ, methyl jasmonate; pI, isoelectric points; TF, transcription factor

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in-combination has been tested as an alternative method for higher production of tanshinones. For example, the introduction of *SmHMGR*, *SmDXS2*, *SmGGPPS* or co-expression of *SmHMGR-SmGGPPS*, *SmHMGR-SmDXR* and *SmGGPPS-SmDXS2* could elevate concentrations of tanshinones in *S. miltiorrhiza* hairy roots [3,4,15]. Nevertheless, complete downstream committed steps in tanshinone biosynthesis remain unclear, which narrows the application of genetic engineering.

Studies have shown that it is an effective approach to enhance the secondary metabolites content by manipulating transcription factors in plants [16]. With genome and transcriptome sequencing, multiple transcription factors (TF) have been identified in *S. miltiorrhiza* including bHLH, MYB, AP2/ERF and the WRKY family [17–20], and the function of several transcription factors have been validated. For example, overexpression of *SmMYC2* could increase the production of phenolic acids in transgenic *S. miltiorrhiza* seedlings [5]. Introduction of the *SmERF11* transcription factor positively regulated tanshinone accumulation in Danshen hairy roots [11], while *SmERF115* played an important role in phenolic acid production [21]. A R2R3-MYB gene *SmMYB9b* has been reported to improve tanshinone production in hairy roots [22], whereas relatively few studies have reported on the regulatory function of WRKY transcription factors in Danshen.

As one of the largest transcription factor families involved in plant kingdom, WRKY transcription factors play a broad role in plants [20,23]. WRKY transcription factors appear to have a common feature which is the WRKY domain that consists of 60 amino acids, with highly conserved WRKYGQK motif located in the N-terminus, and a non-typical zinc-finger-like motif composed of C2HC (C-X₇-C-X₂₃-H-X₁-C) or C2H2 (C-X₄-5-C-X₂₂-23-H-X₁-H) at the C-terminal [20,23–25]. According to the number of WRKY domains and zinc-finger-like motifs, WRKY transcription factors can thus be divided into three subfamilies [20,23,24], and multiple studies have shown that the WRKY transcription factors can bind to the W-box (TTGACC/T) element, located in the promoter regions of the targeted genes [20,23,26–29]. Recently, dozens of studies focusing on WRKY transcription factors have shown their function in metabolism, signal processes and stress responses in many plant species, including traditional Chinese herbal plants [20,28–30]. For example, a group I WRKY from cotton named *GhWRKY25* exhibited enhanced sensitivity to the fungal pathogen *Botrytis cinerea* in *Nicotiana benthamiana* [27]. A WRKY transcription factor GSW1, specifically expressed in the glandular trichomes of *Artemisia annua* could enhance the levels of artemisinin and dihydroartemisinin acid through activating *CYP71AV1* and *AaORA* expression as it binds to the W-box in promoter regions [29]. The *Catharanthus roseus* *WRKY1* positively regulated the accumulation of terpenoid indole alkaloids (TIAs) in hairy root lines [30]. However, there are limited reports on the effects of WRKYs related to secondary metabolism in the model traditional herbal plant, *S. miltiorrhiza* and this presents with opportunities to further resolve the action of WRKYs in this important medicinal species.

In this study, we thus cloned and explored the function of MJ-inducible *SmWRKY2* in *S. miltiorrhiza*. Multiple sequence analysis revealed that it contained two WRKY domains belonging to subgroup I of the WRKY family. Phylogenetic tree analysis showed that *SmWRKY2* had high homology with *AtWRKY4* that is found in *Arabidopsis thaliana*. We thus report on the overexpression of *SmWRKY2* in *S. miltiorrhiza*, using hairy roots as system to explore the biosynthesis of tanshinones. This study also examined the effect of antisense-*SmWRKY2* hairy root transgenesis on tanshinone metabolism. This present study reveals that *SmWRKY2* may be a positive regulator participating in the biosynthesis of tanshinones by increasing the expression of *SmDXS2* and *SmCPS*, which provides a new perspective for tanshinone biosynthesis.

2. Materials and methods

2.1. Plant samples and reagents

S. miltiorrhiza seedlings used for *Agrobacterium*-mediated transformation were cultivated in Murashige and Skoog (MS) medium (pH 5.8) containing 3% (w/v) sugar and 0.8% (w/v) agar and growth conditions were as follows: 16 h light/8 h dark cycle under 25 °C with 60% relative air humidity as reported before [3,4,21]. Seeds of *N. benthamiana* were sown and cultivated in the pots supplemented with soil matrix for 4–5 weeks for infiltration [15,32] and all plants were maintained in the greenhouse.

All strains and plasmid vectors used in this study are part of the collection that is available in our laboratory. The intermediate cloning vector pMD-18 T and reverse transcriptase M-MLV were purchased from TaKaRa Biotechnology (Dalian, China) Limited Company. Primer synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnological Company, China. The RNA extraction kit and qRT-PCR kit were purchased from Tiangen Biotechnology (Beijing) Limited Company, China. Standards of cryptotanshinone, tanshinone I, tanshinone IIA, dihydrotanshinone used for HPLC analysis were purchased from Aladdin, China.

2.2. Elicitor preparation

For MeJA induction, the MeJA (bought from Sigma) was firstly dissolved in a small volume of 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 the elicitation assay, and an equivalent volume of sterilized water was used as the mock treatment [9,37]. Time points of analysis were selected at 0, 0.5, 1, 2, 4 h after MJ treatment to monitor the effects of MeJA to induce metabolite biosynthesis.

2.3. Identification and cloning of *SmWRKY2*

A local reference transcript database of *S. miltiorrhiza* built up as reported previously was used for this research [11]. We obtain the full-length cDNA sequence of the putative *SmWRKY2* gene. Primer pairs for *SmWRKY2-KF* and *SmWRKY2-KR* were synthesized for amplification of the full-length ORF of *SmWRKY2* according to the procedure as described below: initial denaturation at 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. All primers used for identification of *SmWRKY2* are listed in Supplemental Table 1.

2.4. Bioinformatics analysis of *SmWRKY2*

Characterization of *SmWRKY2* were further analyzed by a series of bioinformatics tools. Nucleotide blast, protein blast and ORF Finder were used to assess nucleotide sequences and complete open reading frame (ORF) of the gene of interest. MEGA 6 was applied to construct a phylogenetic tree by the neighbor-joining (NJ) method and 1000 replications were performed for bootstrap values. Multiple sequence alignment of *SmWRKY2* with other WRKYs were carried out using Clustal X with default parameters [32,38]. The isoelectric point was predicted online (<https://web.expasy.org/protparam/>).

2.5. Expression pattern of *SmWRKY2*

Different tissues including the tap root, stem, leaf, flower and seed were gathered from two-year-old *S. miltiorrhiza* plants. Elicitor treatments were conducted on *S. miltiorrhiza* hairy roots derived from Ri plasmid harbouring C58C1 strain of *Agrobacterium tumefaciens* transformation that are routinely subcultured every 60 days. Hairy roots were harvested at selected time points (0, 0.5, 1, 2 and 4 h) after MJ

treatment. All the treated samples were immediately frozen in liquid nitrogen and stored at -80°C before the analysis of the expression profiles of *SmWRKY2* in generated transgenic lines.

2.6. Subcellular localization of *SmWRKY2*

To analyze the subcellular localization of *SmWRKY2*, PCR products of the full-length of *SmWRKY2* with *Hind* III and *Spe* I restriction sites were cloned into the vector pHB-YFP to generate the recombinant vector pHB-*SmWRKY2*-YFP. The constructed expression vector was transferred into *Agrobacterium tumefaciens* strain GV3101 and injected into forty-day-old tobacco leaves [29,32,38]. DAPI solution of $1\ \mu\text{g mL}^{-1}$ was injected into *N. benthamiana* leaves 3 h before observation. YFP fluorescence was observed after 48 h cultivation using a confocal microscope (Carl Zeiss).

2.7. Generation of transgenic *SmWRKY2* hairy roots

The full-length coding sequence of *SmWRKY2* with restriction sites, *Spe*I and *Bst*E II, was cloned and inserted into a modified pCAMBIA2300sm vector (where the small fragment digested by *Eco*R I and *Hind* III is replaced with the corresponding GFP-GUS gene expression cassette from pCAMBIA1304) under the control of the CaMV 35S promoter to generate pCAMBIA2300sm-*SmWRKY2* as described before (Fig. S2) [11,38]. The disarmed *A. tumefaciens* strain C58C1 harboring the *A. rhizogenes* Ri plasmid pRiA4 and pCAMBIA2300sm-*SmWRKY2* was used to infect the aseptic explants, generated via tissue culture, and the empty pCAMBIA2300sm was regarded as the control. The transformation procedure was the same as our previous study [3,4,11,38]. Those hairy roots that showed a good hairy root phenotype were subcultured and primer pairs CaMV35S-F23 and *SmWRKY2*-QR were used to identify the positive lines by polymerase chain reaction (PCR) analysis, and the *rol* B gene was also detected. Genomic DNA was extracted from hairy root lines using the cetyltrimethyl ammonium bromide (CTAB) method as reported previously [32,39]. Identified positive clones were cut into segments that were approximately 4 cm long for shake-flask culture in 100 mL 1/2MS medium and these were cultured at 25°C on an orbital shaker shaking at the speed of 100 rpm in the dark [15,38]. Primers sequences used for amplifications are listed in Supplemental Table 1.

2.8. Total RNA isolation and relative expression analysis via qRT-PCR

Expression profiles of *SmWRKY2* and several key enzyme genes involved in the tanshinone 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 [3,15]. Total RNA served as the template for reverse transcription (RT) reaction, the reaction conditions were according to our previous studies [9,15,38]. Gene-specific primers (listed in Supplemental Table 1) for qRT-PCR were designed to assay the relative expression level and were compared with an internal reference gene *SmActin*, using the relative quantitative analysis method ($2^{-\Delta\Delta\text{CT}}$). Amplifications were performed according to the manufacturer's instructions: one cycle of denaturation at 95°C for 15 min, then 40 cycles of 15 s denaturation at 95°C , 30 s annealing at 60°C and 30 s extension at 72°C [11,21].

2.9. Dual-Luciferase (Dual-LUC) assay

For the dual-LUC assay, the promoters of *SmDXS2* and *SmCPS* with *Kpn*I and *Xho*I restriction sites were cloned into pGREEN0800 to drive the luciferase reporters, respectively. And the complete ORF of *SmWRKY2* was inserted into the pHB-YFP vector as effector. The pHB-*SmWRKY2*-YFP and pHB-YFP empty plasmid were transferred into *Agrobacterium tumefaciens* strain GV3101 individually. The

pGREEN0800-p*SmDXS2*, pGREEN0800-p*SmCPS* was separately co-transformed with the helper plasmid pSoup19 into GV3101, and the assay was conducted as described before [11,21,29]. The pHB-YFP 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).

2.10. Yeast one-hybrid assay

We performed yeast one-hybrid assay as reported before [29]. The coding sequence of full-length *SmWRKY2* cDNA was fused into the pB42AD vector. *SmCPS* promoter sequence (from -323 bp to -329 bp, relative to translation start site) was inserted into a p*LacZ* plasmid. The resulting recombinant plasmids were co-transformed into yeast strain EGY48a. The transformants were cultivated on SD/-Ura/-Trp medium for two days, and tested on SD/-Ura/-Trp medium with 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) for 24 h. The empty vectors pB42AD and p*LacZ* were used as negative controls.

2.11. Electrophoretic mobility shift assay (EMSA)

The ORF of *SmWRKY2* was inserted into the expression vector p*ET28a* between the *Nco*I and *Sal*I sites. Recombinant *SmWRKY2* was expressed in *Escherichia coli* strain BL21, and purified using a 6×HIS-Tagged Protein Kit (Cwbio, Shanghai, China). Oligonucleotide probes for the *SmCPS* promoter was labeled with an EMSA probe biotin labeling kit (Beyotime, Haimen, China), following the manufacturer's instructions. The DNA fragment and purified proteins were incubated in $10 \times$ EMSA binding buffer (2 mM DTT, 0.2 mM EDTA, 50 mM HEPES-KOH at pH 7.6, 300 mM NaCl, $100\ \text{ng}\ \mu\text{L}^{-1}$ poly (dI-dC), and $400\ \text{ng}\ \mu\text{L}^{-1}$ salmon sperm DNA) at 25°C for 20–25 min. The DNA-protein complexes were then electro-transferred and detected with a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Shanghai, China), following the manufacturer's instructions. Fluorescence was detected with a c300 image scanner (Azure Biosystems, USA). The probe sequences are listed in Supplemental Table 1.

2.12. HPLC analysis of tanshinones

The 60-day-old hairy roots were dried at 50°C to a constant weight in an oven. For each analysis, approximately 100 mg dried hairy roots were ground into a powder and immersed in 16 mL methanol/dichloromethane (3:1, v/v) for tanshinone extraction. Tanshinone extractions were carried out as reported before [4,9,11]. HPLC analysis was performed on an Agilent 1260 apparatus equipped with a Waters reversed-phase C18 symmetry column, and the detection conditions were performed following the methods described previously [3,15,38], which worked as the mobile phase at a flow rate of 1 mL/min with the detection wavelength at 270 nm. Total tanshinones was regarded as TT in this study.

2.13. Statistical analysis

All the experiments including culture of hairy root clones, PCR identification, qRT-PCR and HPLC analysis were repeated three times. Results of tanshinone content were presented as mean values \pm SD. The error bars were from biological triplicates. The statistically significant difference was analyzed by one sample *t* test, and the difference of various hairy root lines were used in the one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS, Inc.).

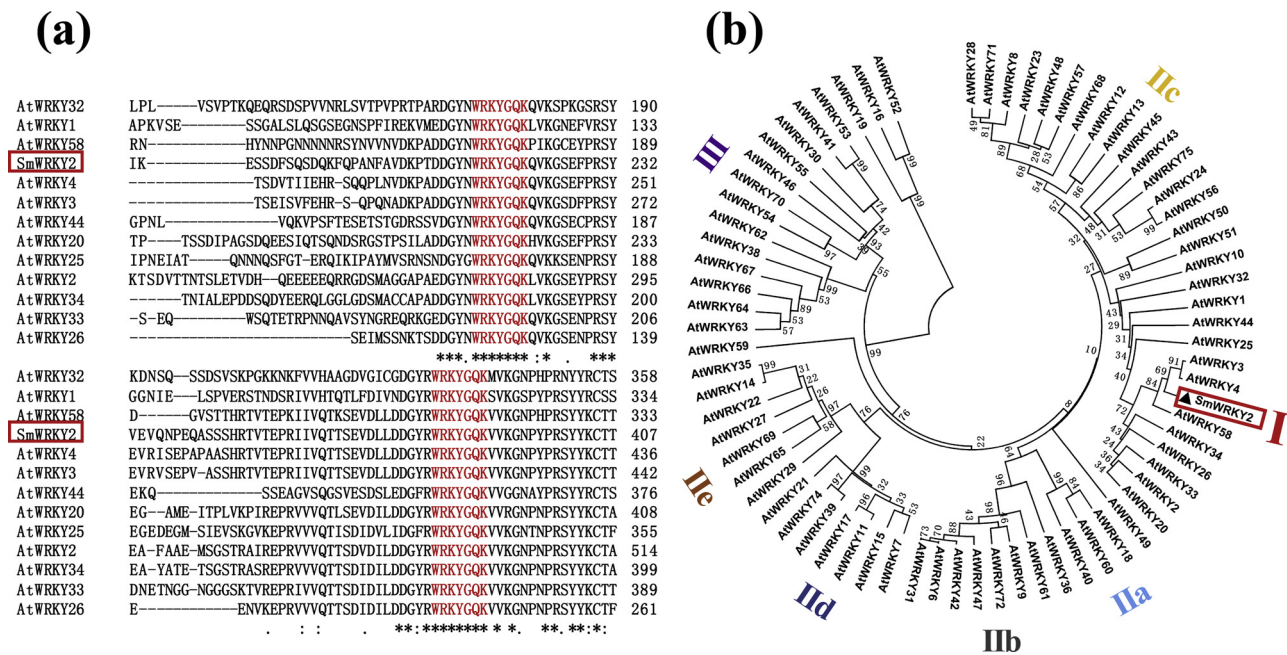


Fig. 1. Bioinformatics analysis of SmWRKY2.

(a) Multiple alignment of SmWRKY2 with related WRKY proteins from *Arabidopsis thaliana*. Red regions indicate that they have the same conserved domain, which is WRKYGQK. (b) Phylogenetic tree analysis of SmWRKY2 and WRKY transcription factors from *Arabidopsis thaliana*. The phylogenetic tree was constructed on MEGA6.0 by using the neighbor-joining method and the bootstrap values were obtained for 1000 replications. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

3. Results

3.1. Isolation and characterization of SmWRKY2

By searching our local transcriptome database [19], one WRKY showing highly homology to WRKY4 from *Arabidopsis thaliana* (AtWRKY4) was cloned for further research. The putative SmWRKY2 ORF contains 1476 bp and encodes a protein of 491 amino acids with a calculated molecular mass of 53.3KD and pI of pH 7.26. To further figure out the biological characteristics and phylogenetic relationship of SmWRKY2, a series of bioinformatic analyses were performed. Multiple alignment of SmWRKY2 with related WRKY proteins from *A. thaliana* revealed that SmWRKY2 contained two conserved WRKY domain (WRKYGQK) and a special zinc finger like motif in its C-terminal which falls into the group I of WRKY family (Fig. 1a). Phylogenetic analysis illustrated that SmWRKY2 has a close relationship with AtWRKY4 (Fig. 1b).

3.2. Expression patterns of SmWRKY2

To investigate the tissue expression pattern of SmWRKY2, different tissues including root, stem, leaf, flower and seed from two-year-old *S. miltiorrhiza* plants were analyzed. SmWRKY2 showed high expression in leaf and stem tissues, and relatively low expression level in the flower and roots, plus its transcript was barely detected in seed (Fig. 2a). This result indicated that SmWRKY2 does not exhibit a tissue-constitutive gene expression. The elicitation results indicated that SmWRKY2 expression was induced by exogenous MeJA with the expression levels reaching a peak at 1 h after treatment, leading to an approximate 2-fold increase compared with control. This was followed by another increase at 4 h (Fig. 2b), suggesting that SmWRKY2 may participate in MJ signalling.

3.3. Subcellular localization of SmWRKY2

To experimentally confirm the subcellular localization of

SmWRKY2, SmWRKY2 was cloned into the pHB vector fused with a yellow fluorescent protein (YFP) reporter gene to generate the vector pHB-SmWRKY2-YFP. Then, the constructed vector and the pHB-YFP (used as the control) was transformed into GV3101 strain and expressed in tobacco leaves, respectively. Fluorescence of YFP was detected in the cytoplasm and nucleus in the tobacco leaves transformed with the empty vector. Contrast to this, the fluorescent signal of SmWRKY2 was only detected in the nucleus (Fig. 3). The expression pattern was consistent with the characteristic mode of action of SmWRKY2 as a transcription factor.

3.4. Generation of SmWRKY2 transgenic hairy roots

To further investigate the function of SmWRKY2 in *S. miltiorrhiza*, we inserted SmWRKY2 into a modified pCAMBIA2300sm vector. Afterwards, the recombinant overexpression vector pCAMBIA2300sm-SmWRKY2 was introduced into the modified *Agrobacterium tumefaciens* strain C58C1 and used to infect *S. miltiorrhiza* explants, while the empty vector pCAMBIA2300sm was used as control. After the infection process, fresh hairy roots could be induced from stem or leaf explants (Fig. 4a). The positive lines carrying the SmWRKY2 gene (OE line) were verified by genomic PCR and a positive transformation rate of 26.92% among the 21 hairy root lines was obtained. And 50% positive rate of anti-sense lines were generated. Three individual over-expressed (OE-1, 2, 3) and two anti-sense lines (Anti-sense-1, 2) were chosen for further study (Fig. 4b). qRT-PCR analysis of the expression of SmWRKY2 in overexpression lines revealed that SmWRKY2 was expressed 5 to 65-fold higher than in the control lines, while the expression is slightly lower in the Anti-SmWRKY2 lines than the controls (Fig. 5a).

3.5. SmDXS2 and SmCPS were significantly up-regulated by SmWRKY2 in vivo

To study whether SmWRKY2 participated in the regulation of tanninone biosynthesis, transcript levels of several genes related to tanninone biosynthesis in SmWRKY2 transgenic hairy root were analyzed

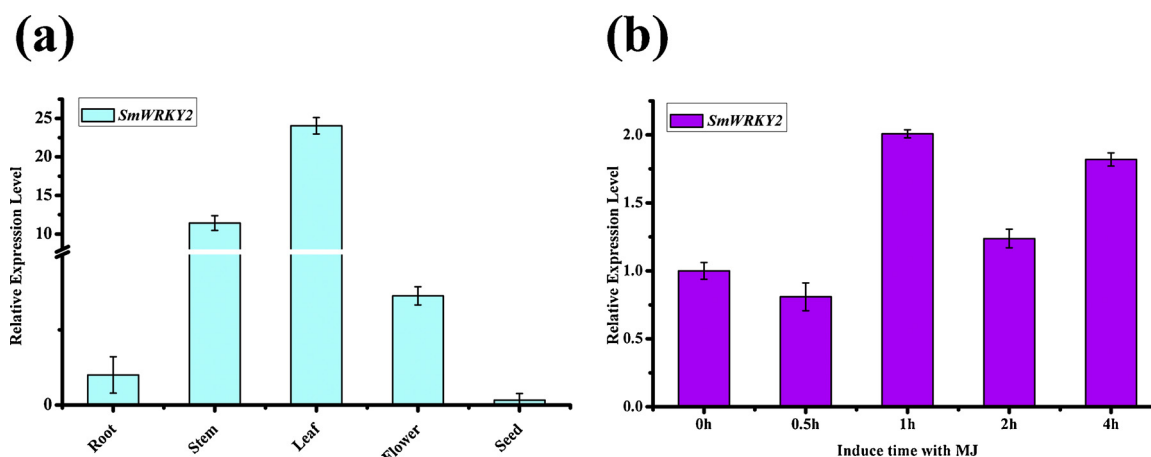


Fig. 2. Expression pattern of *SmWRKY2*.

(a) Expression pattern of *SmWRKY2* in different tissues. Each tissue was obtained from several individual 2-year-old *S. miltiorrhiza* plants in nature. (b) The expression level of *SmWRKY2* after MJ treatment for selected time points measured by qRT-PCR. Values are means \pm standard deviation of triplicate analyses, transcript abundance of *SmWRKY2* is normalized to the internal standard *SmActin* using the $2^{-\Delta\Delta CT}$ method.

by qRT-PCR. Several tanshinone biosynthesis pathway genes were up-regulated in the *SmWRKY2*-overexpressing hairy roots, the most striking ones were *SmDXS2* and *SmCPS*, resulting in 10–15 folds and 5–25 folds increases compared with the control, respectively. Meanwhile the expression of *SmIPPI*, *SmGGPPS*, *SmKSL* and *SmCYP76AH1* was also increased by about 2–4 folds in overexpression lines than the control, whereas, only *CPS* was slightly downregulated in antisense lines (Fig. 5b). Dual-Luciferase (Dual-LUC) assay showed that *SmWRKY2* can elevated the expression of *SmDXS2* and *SmCPS* about 2–3 folds. The data revealed that *SmWRKY2* may be a regulator in tanshinone biosynthesis by positively activating the expression of *SmDXS2* and *SmCPS* (Fig. 6b, Fig. S3b).

3.6. Activation of the expression of *SmCPS* by *SmWRKY2*

To investigate further how *SmWRKY2* regulates tanshinone biosynthesis, we performed Dual-LUC assays. The result showed that *SmWRKY2* activated the transcription of *SmDXS2* and *SmCPS*, But the *SmDXS2* slightly up-regulated. Meanwhile, analyzed the *SmDXS2* and *SmCPS* promoter sequences and found that they all have W-box (Figs. 6a, S3a). Y1H assay showed that *SmWRKY2* was bound to the W-box of *SmCPS* promoter (Fig. 6c). We performed an EMSA using purified His-*SmWRKY2* recombinant protein and biotin-modified oligonucleotide probes containing the W-box sequences to examine the binding of *SmWRKY2* to the *SmCPS* promoter (Fig. 6d). Our result indicated that *SmWRKY2* could bind to the W-box element of the *SmCPS* promoter region, which was consistent with that *SmCPS* expression was

effectively activated in *SmWRKY2*-overexpressing *S. miltiorrhiza* hairy roots.

3.7. Accumulation of tanshinone was obviously affected by *SmWRKY2*

Four kinds of tanshinones including cryptotanshinone, dihydrotanshinone I, tanshinone I, tanshinone IIA were examined in hairy roots by HPLC. The results showed that the content of total tanshinones were significantly up-regulated from 3.003 to 3.985 mg/g DW in over-expressed lines. Among them, *OE-SmWRKY2-1* line accumulated the highest content of total tanshinone, which was 1.83 times as that of the control. The total tanshinone content in the two antisense-*SmWRKY2* lines was slightly lower than the control group (Fig. 7). These results further confirmed the positive role of *SmWRKY2* in the regulation of tanshinone biosynthesis.

4. Discussion

S. miltiorrhiza is a traditional Chinese herbal medicine that has been used for more than two thousand years [14]. Previous studies have shown that tanshinones are the main medicinal active ingredient of *S. miltiorrhiza* that can be used to treat cardiovascular and cerebrovascular diseases [31]. However, traditional *S. miltiorrhiza* production cannot meet the growing clinical needs due to its slow growth, low tanshinone content and scarcity of wild resources [32] and for these reasons biotechnological approaches may provide alternative means for tanshinone production. The diterpenoid tanshinone was synthesized in

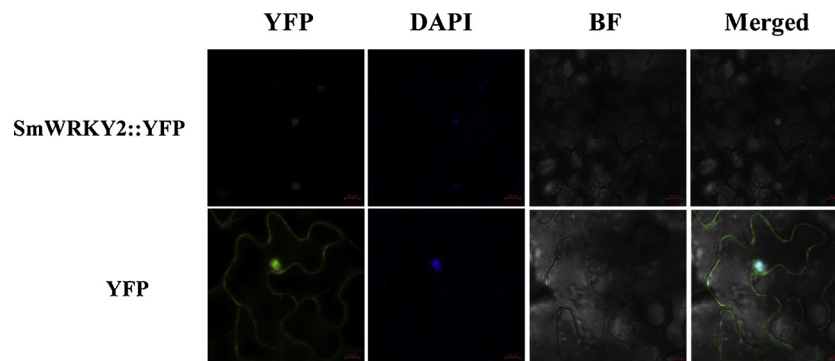


Fig. 3. Subcellular localization of *SmWRKY2*.

Subcellular localization of *SmWRKY2* in *N. benthamiana* leaves. In the upper row, *SmWRKY2*::YFP expressed in the nucleus under the control of CaMV35S promoter. In the lower row, YFP control expressed in the whole *N. benthamiana* leaf cell.

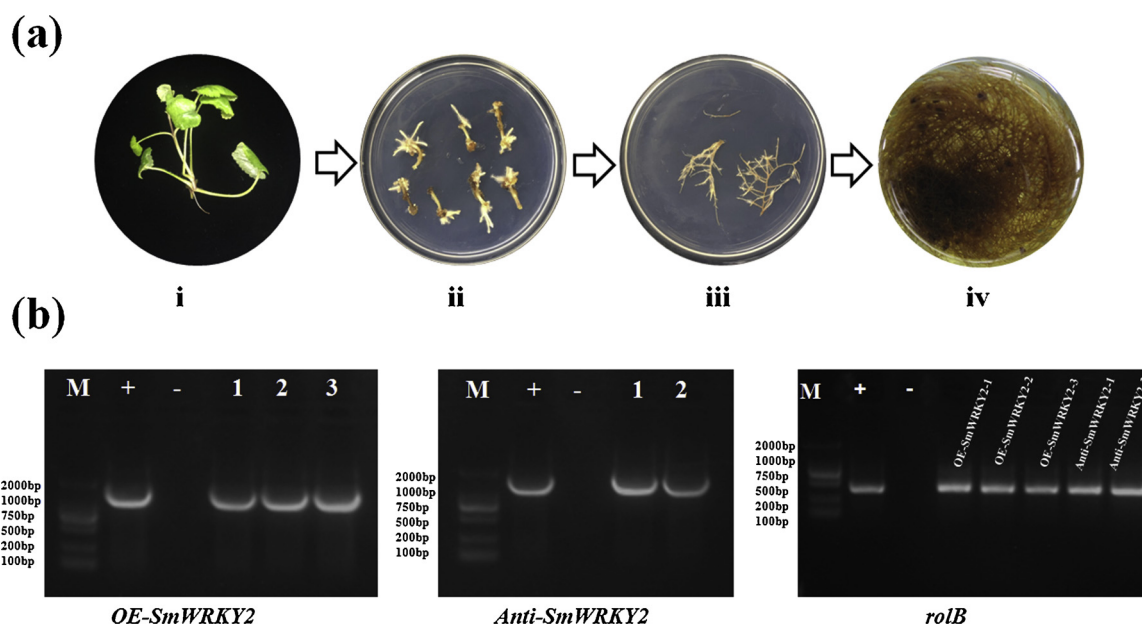


Fig. 4. Acquisition and identification of hairy roots transgenic hairy roots lines.

(a) Generation of transgenic hairy root of *S. miltiorrhiza*. i) *S. miltiorrhiza* seedlings in MS medium; ii) growing hairy roots on the infected *S. miltiorrhiza* explants; iii) a single clone of a hairy root line; and iv), hairy root cultured in 1/2 MS medium. (b) Identification of positive transgenic hairy root lines by PCR.

distinct pathways including the MVA and MEP pathways, and several key genes have been isolated in Danshen. For example, DXS catalyzes the first step in MEP pathway by metabolizing pyruvate and glyceraldehyde 3-phosphate to form DXP, which has been validated as a rate-limiting process [3]. RNA interference of *SmCPS* expression resulted in reduced tanshinone content in transgenic hairy roots [33], suggesting that *SmCPS* is a key enzyme in tanshinone biosynthesis. Genetic engineering of key enzyme genes individually or in combinations has become an effective and important way to increase the accumulation of active ingredients in *S. miltiorrhiza* [3]. For example, introduction of single *SmDXS2* gene in danshen hairy roots produced tanshinone quantities ranging from 2.02–4.58 mg/g DW, which was much higher than the control line [11]. Co-expression of *SmGGPPS* and *SmDXS2* led to superior tanshinone production, recorded at 12.93 mg/g DW [11].

WRKY is one of the largest plant transcription factor families that have been studied recently [28,30]. The conserved domain WRKYGQK and the zinc finger motif consisting of 60 amino acids is considered as a

general feature of WRKY transcription factors and can also serve as a standard means to identify this subgroup. Previous studies have demonstrated that WRKY transcription factors can directly bind to W-box of related genes from different signaling pathways and play a regulatory role in plant stress tolerance [23,26,27]. For example, *SpWRKY1* has been shown to promote resistance to *Phytophthora nicotianae* and tolerance to salt and drought stress in transgenic tobacco [25]. Over-expression of *NtWRKY50* increased the expression level of related defense genes and improve resistance to *Ralstonia solanacearum* in tobacco [34]. *GhWRKY25* (member of Group I) from cotton confers differential tolerance to non-biological and biotic stress in transgenic tobacco [27]. In recent years, there is increasing evidence that WRKY transcription factors are not only involved in plant stress resistance, but also in plant secondary metabolism regulation [28,30]. For example, *Taxus chinensis WRKY1* may enhance the expression of 10-deacetylbaicatin III-10 β -O-acetyl transferase (DBAT) to increase the accumulation of taxol in *Taxus* cell cultures [35]. A WRKY gene named *WRKY1* from *Withania*

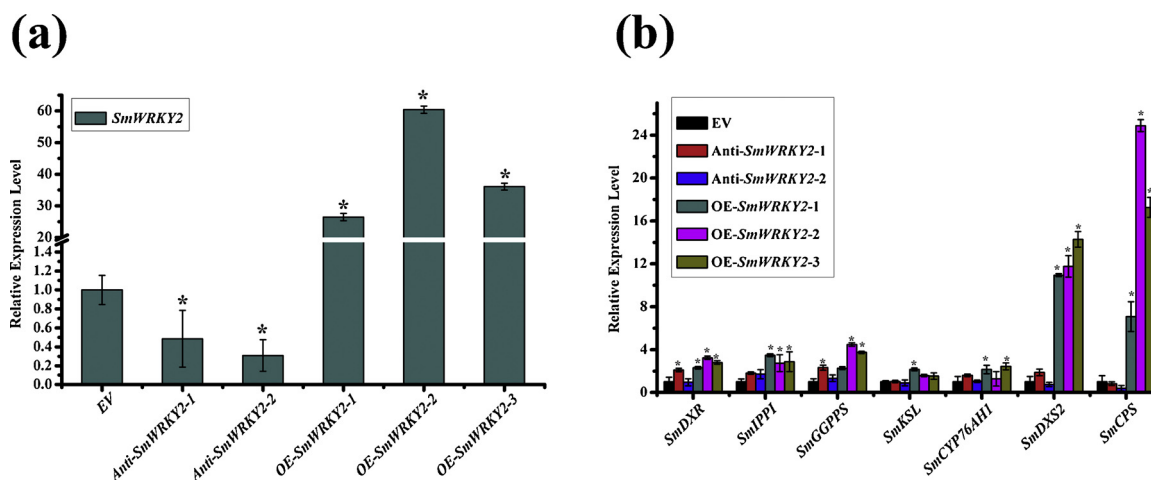


Fig. 5. Detection of transcript level of rate-limiting enzyme genes.

(a) Transcript levels of *SmWRKY2* in transgenic hairy roots detected by qRT-PCR. (b) Transcript levels of key tanshinone biosynthesis genes in *SmWRKY2* transgenic hairy roots, indicating that *SmDXS2* and *SmCPS* are significantly up-regulated. All data are means of three replicates, with error bars indicating standard deviations. The values are means \pm S.D of triplicate analysis ($P < 0.05$).

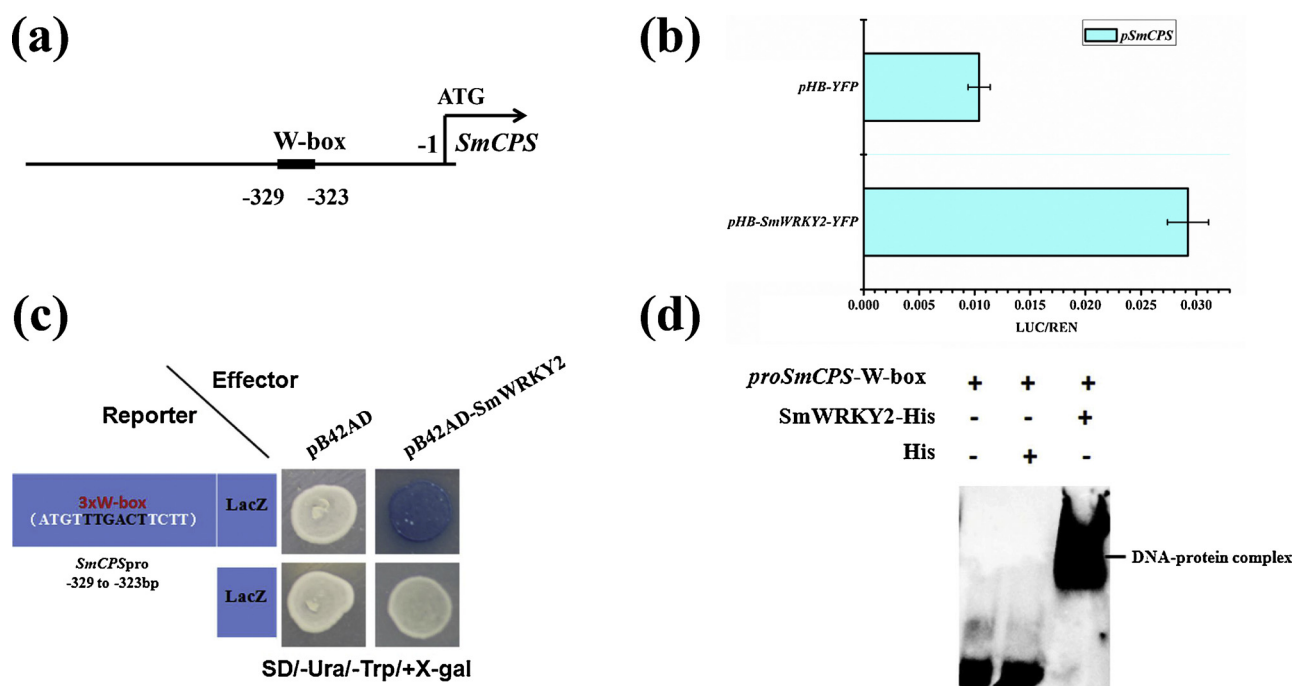


Fig. 6. *SmWRKY2* activates the transcription of *SmCPS* *in vivo*.

SmWRKY2 binds to the W-box of the *SmCPS* promoter and activates *SmCPS* expression. (a) The analysis of motifs in *SmCPS* promoter. (b) The Dual-luciferase assay that *SmWRKY2* activates the promoter of *SmCPS*. The *SmCPS* 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. (c) Yeast one-hybrid assay showing that *SmWRKY2* binds to the W-box of *SmCPS* promoter. (d) EMSA showing that *SmWRKY2* binds to the W-box of the *SmCPS* promoter. The His-*SmWRKY2* fusion protein and labeled probe were used. The His protein was included as a negative control. Error bars indicate SD (n = 3).

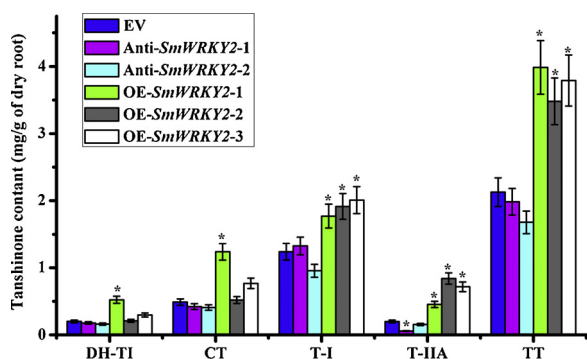


Fig. 7. HPLC analysis of tanshinones in transgenic lines.

The production of tanshinone in *SmWRKY2* transgenic hairy roots compared with control line detected by HPLC. Three overexpressed lines (*OE-SmWRKY2-1*, *2*, *3*) and two antisense lines (*Anti-SmWRKY2-1*, *2*) were used to compare with the control hairy root line. DH-TI, dihydrotanshinone; CT, cryptotanshinone; T-I, tanshinone I; T-IIA, tanshinone IIA; TT represented total tanshinone. All data are means of three replicates, with error bars indicating standard deviations. The values are means \pm S.D of triplicate analysis ($P < 0.05$).

somnifera could target squalene synthase and squalene epoxidase gene for improvement of triterpenoid withanolide accumulation [36]. However, studies on the function of WRKY transcription factors in *S. miltiorrhiza* regulating the biosynthesis of tanshinones are relatively rare.

In our study, a novel WRKY transcription factor *SmWRKY2* was successfully cloned from *S. miltiorrhiza*, with high homology to *AtWRKY4* factor from *Arabidopsis*. In *S. miltiorrhiza*, exogenous MeJA treatment can promote the accumulation of tanshinone [9,37]. In our study, we noted that *SmWRKY2* can be induced by exogenous MeJA treatment, reaching high levels of accumulation 1 h after the MeJA

treatment, which is consistent with previous reports that MeJA treatment can increase tanshinone production [9,19]. Analysis using HPLC showed that the introduction of *SmWRKY2* in transgenic hairy roots increased the tanshinone to 3.985 mg/g DW, which was 1.83 times as that of the non-transgenic line. Quantification via qRT-PCR analysis showed that *SmWRKY2* overexpression significantly promoted the transcript levels of *SmDXS2* and *SmCPS* which are two key genes in tanshinone biosynthesis, dual-Luciferase (dual-LUC) assay showed that *SmWRKY2* can positively regulate *SmDXS2* and *SmCPS* expression, Y1H and EMSA experiments indicate that *SmWRKY2* only binds to the W-box of the *SmCPS* promoter, suggesting the regulatory role of *SmWRKY2* in tanshinone synthesis in *S. miltiorrhiza*. The expression profile showed that *SmWRKY2* responded to MeJA, suggesting that *SmWRKY2* may be involved in stress-regulating processes, such as defense against pathogens, but further experimental confirmation is needed to verify this idea. In summary, our work reveals that transcription factor *SmWRKY2* is involved in promotion of tanshinone biosynthesis and provides new insights into the potential of using a genetic engineering strategy to increase the yield of target compounds in *S. miltiorrhiza*.

Author contributions

G.Y.K. conceived and designed the experiments. C.P.D., X.L.H., M.S., R.F., Y.W., Y.Z., and Y.F. performed the experiments. C.P.D., X.L.H. and W.Z. analyzed the data. M.S., X.L.H., C.P.D., N.P.M. and G.Y.K. wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.plantsci.2019.03.007>.

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