Enhancing diterpenoid concentration in *Salvia miltiorrhiza* hairy roots through pathway engineering with maize C1 transcription factor

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Received 30 March 2015; Revised 6 August 2015; Accepted 19 August 2015

Editor: Ian Dodd

Abstract

Tanshinones are valuable natural diterpenoids from danshen (*Salvia miltiorrhiza* Bunge). Here, it was demonstrated that maize transcription factor C1 improved the accumulation of tanshinones by comprehensively upregulating the pathway genes, especially *SmMDC* and *SmPMK* in danshen hairy roots, yielding total tanshinones up to 3.59 mg g\(^{-1}\) of dry weight in line C1-6, a 3.4-fold increase compared with the control. Investigation of 2024 bp of the *SmMDC* promoter fragment revealed that C1-mediated upregulation of terpenoid genes was possibly due to the direct interaction of C1 with its recognition sequences. The increase of tanshinones was accompanied by a decrease of salvianolic acid production, the other bioactive ingredient in danshen, by up to 37% compared with the control. This was the result of the downregulation of *SmTAT*, the entry-point gene of the tyrosine pathway, which promoted metabolic flow to anthocyanins rather than to salvianolic acids. Based on the findings of the present study, it was concluded that cis-acting elements shared by terpenoid and phenylpropanoid biosynthetic genes are partially responsible for the C1-stimulated variation of tanshinone and salvianolic acid concentrations.

Key words: Hairy roots, regulation, *Salvia miltiorrhiza* Bunge, salvianolic acids, tanshinones, ZmC1.

Introduction

Danshen (*Salvia miltiorrhiza* Bunge) is a widely used medicinal herb for the prevention and treatment of cardiovascular and cerebrovascular disorders with lipophilic diterpenoids (tanshinones) and hydrophilic phenylpropanoids (salvianolic acids) as the two major bioactive ingredients (Xu, 1990; Li and Luo, 2008). The representative component of tanshinones is tanshinone IIA (TA-IIA) and that of salvianolic acids is salvianolic acid B (SAB; also named lithospermic acid, LAB) (Li and Luo, 2008). In clinical studies, both compounds have been applied through intravenous injection for the treatment of coronary heart disease and angina (Li and Luo, 2008).

Tanshinones are a series of abietane-type diterpenoids that are synthesized through the terpenoid metabolism including the cytoplasmic mevalonate (MVA) pathway and the plastidic 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway. The MVA pathway starts with the condensation of three acetyl-CoA units to MVA successively catalysed by acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl
CoA synthase (HMGS), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). MVA is phosphorylated and decarboxylated to isopentenyl pyrophosphate (IPP) by mevalonate kinase, 5-phosphomevalonate kinase (PMK) and mevalonate pyrophosphate decarboxylase (MDC) in turn (Okada, 2011). The MEP pathway begins with the coupling of one molecule of pyruvate with one molecule of glyceraldehyde-3-phosphate to IPP through seven enzymatic steps that include 1-deoxy-d-xylulose 5-phosphate synthase (DXS), 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), 2-C-methyl-d-erythritol 4-phosphate cytidylyltransferase (MCT), 4-(cytidine-5’-diphospho)-2-C-methyl-d-erythritol kinase (CMK), 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS), 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) (Xu et al., 2010; Kai et al., 2011; Ma et al., 2012; Yang et al., 2013). After it has been synthesized, IPP may be transformed to dimethylallyl diphosphate (DMAPP) and transported within the cytosol and the plastid freely. Like the formation of most other terpenoids, tanshinones are produced through the condensation of IPP with DMAPP followed by sequential cyclization and dehydrogenation reactions (Fig. 1).

Salvianolic acids originate from the phenylpropanoid acid metabolism (Fig. 1) (Petersen et al., 2009; Di et al., 2013; Ma et al., 2013; Petersen, 2013; Zhang et al., 2014). The general phenylpropanoid pathway provides 4-coumaroyl-CoA as one active precursor synthesized from L-phenylalanine by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumaric acid CoA-ligase (4CL); the tyrosine biosynthetic pathway offers the other precursor, 4-hydroxyphenylacetic acid (4-HPLA) synthesized from L-tyrosine by tyrosine amino transferase (TAT) and hydroxylphenylpyruvic acid reductase (HPPR). These two active precursors are then coupled together by rosamarinic acid synthase (RAS) and hydroxyl groups are introduced by a cytochrome P450-dependent monoxygenase (CYP98A14) cooperating with NADPH:cytochrome P450 reductase (CPR) to form

![Diagram of biosynthetic pathway of phenylpropanoids and terpenoids from the primary metabolism in S. miltiorrhiza](https://academic.oup.com/jxb/article-abstract/66/22/7211/2893282/fig1)
rosmarinic acid (RA) (Eberle et al., 2009; Petersen et al., 2009; Di et al., 2013; Petersen, 2013). SAB and its structural related phenolic acids are proposed to be the condensation derivatives of RA (Di et al., 2013).

With the elucidation of the biosynthetic pathway, metabolic engineering has been employed to manipulate the metabolic flux and explore the regulatory networks involved. The overexpression of single C4H, TAT, or HPPR, or both the TAT and HPPR genes allowed danshen hairy roots to accumulate RA and SAB at up to 906 mg l\(^{-1}\) and 992 mg l\(^{-1}\), respectively, proving that these enzymes play key roles in the biosynthesis of salvianolic acids (Xiao et al., 2011). Ectopic expression of Arabidopsis transcription factor Production of Anthocyanin Pigment 1 (AtPAPI), an R2R3-MYB transcription factor gene, resulted in an engineered S. miltiorrhiza plant producing about 73 mg g\(^{-1}\) of dry weight (DW) of SAB, a 2-fold increase compared with the wild-type production (Zhang et al., 2010). Combining the expression of AtPAPI with suppression of both cinnamoyl-CoA reductase (SmCCR) and caffeic acid O-methyltransferase (SmCOMT), the resulting transgenic plant increased by 3-fold the content of salvianolic acids along with a reduced lignin concentration, demonstrating that heterologous transcription factors could modulate the biosynthesis of natural products (Zhang et al., 2014). In contrast to the work on salvianolic acids, metabolic engineering of tanshinone production has been limited. By overexpressing SmHMG and SmGGPPS, the engineered danshen hairy root HG9 yielded up to 2.7 mg g\(^{-1}\) of DW of tanshinones, a 4.74-fold increase compared with the promoter (Kai et al., 2011). However, to the best of our knowledge, there have been no prior literature reports about the correlation between the biosynthesis of salvianolic acids and tanshinones or reports about the simultaneous engineering of both pathway genes in S. miltiorrhiza until now.

Maize (Zea mays) C1 and R are well-elucidated R2R3-MYB and basic helix-loop-helix (bHLH) MYC-type transcription factors, respectively. They could regulate the biosynthesis of anthocyanins and flavonoids (Bovy et al., 2002; Grotewold et al., 1998). Ectopic expression of C1 and R in maize Black Mexican Sweet suspension cells led to the accumulation of reddish-purple cyanidin derivatives (Grotewold et al., 1998). Combination of expression of maize C1 and Lc, a member of the MYC-type R transcription factors, upregulated the flavonoid pathway and resulted in high flavonol accumulation in tomato fruit flesh (Bovy et al., 2002).

This study reports the application of maize C1 for engineering the production of secondary metabolites in danshen hairy roots. The putative mechanism of C1-mediated upregulation of terpenoid pathway genes was explored. This work resulted in the highest tanshinone-producing hairy roots reported to date, providing evidence for possible crosstalk between the terpenoid and phenylpropanoid metabolism in S. miltiorrhiza. (Hu and Alfermann, 1993). For the biomass, compound, and gene expression assays, 0.5 g of fresh hairy root segments were inoculated into 250 ml flasks containing 100 ml of liquid medium and cultured in a shaker at 100 rpm in the dark at 25 °C for 3 weeks.

DNA and RNA preparation

Genomic DNA was isolated from fresh hairy roots using the cetyl trimethylammonium bromide method, checked for integrity with agarose gel electrophoresis and quantified using a spectrophotometer. Total RNA was isolated from fresh hairy roots using the phenol/chloroform guanidinium thiocyanate method and cDNA synthesis was performed using 2 μg of total RNA per 25 μl reaction after being treated with RNase-free DNase I (Sambrook and Russell, 2001).

Plant expression vector construction

The pCAM-35S::C1 and pCAM-35S::R expression constructs were created by replacing the mGFP part of pCAMBIA1302 (http://www.cambia.org). Last accessed 27 August 2015) with C1 or R coding regions. The pCAM-35S::C1-35S::R binary vector was generated by inserting the 35S::R-NOS fragment into the multiple cloning site of the pCAM-35S::C1 construct. A schematic diagram of the constructs is shown in Supplementary Fig. S1, available at JXB online. The empty pCAM vector was used as the control. All constructs were separately introduced into Agrobacterium rhizogenes LBA9402 for induction of transgenic hairy roots.

For the construction of plant transient expression vectors, the C1 ORF in pCAM-35S::C1 was firstly replaced with the firefly luciferase ORF region leading to p35S::Luc. Then, the cauliflower mosaic virus (CaMV) 35S promoter was replaced with the truncated SmMDC promoter fragments, yielding pF1::Luc, p5F1::Luc, and pF2::Luc vectors. Renilla luciferase gene was inserted into p35S::Rluc for co-transformation to calibrate the reading of firefly luciferase. These constructs were introduced separately into Agrobacterium tumefaciens GV3101 for transient expression assay. All constructs were confirmed by DNA sequencing.

Generation of danshen transgenic hairy roots and cells

Hairy roots were induced as described previously (Hu and Alfermann, 1993). After infection with A. rhizogenes containing pCAM-35S::C1, pCAM-35S::R, pCAM-35S::C1-35S::R, or pCAM constructs, danshen leaf discs were inoculated onto MSOH solid medium without any antibiotics for 2 d and then transferred to MSOH liquid medium with the claforan concentration gradually reduced from 250 mg l\(^{-1}\) to zero over three to five generations. PCR (HerogenBio, China) was then performed to select four genotype hairy roots with committed genes, C1, R, or both C1 and R (C1/R) and role, named C1-hairy roots, R-hairy roots, C1/R-hairy roots, and P-hairy roots (the control), respectively (Supplementary Fig. S2, available at JXB online). Transgenic cells were induced from C1-6 transgenic hairy root lines by inoculating the individually wounded hairy roots in MS solid medium with 6-benzaminopurine and kinetin. Control cells were from P16 control hairy root.

Material and methods

Plant materials

Danshen (S. miltiorrhiza Bunge) plants were cultivated in the garden of the Shanghai University of Traditional Chinese Medicine. All hairy roots were cultured in MSOH medium as described previous

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on an ABI StepOne Plus (Applied Biosystems, CA, USA) using SYBR® Premix Ex Taq™ (TaKaRa, China). Gene-specific primers were designed using Primer Premier 6.0 software (Supplementary Table S1, available at JXB online) and those primer pairs with 95–105% amplification efficiency were

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selected for the qRT-PCR assay. To evaluate the dosage of C1 and R, an absolute expression assay was performed. To do this, a standard curve was made using 197 nt of C1 and 154 nt of R purified PCR products. The standard curve for C1 was $y = 3.3086x + 32.396$ ($R^2=0.9994$) and that for R was $y = 3.2389x^2 + 35.283$ ($R^2=0.9985$), where $y$ is the $C_t$ value and $x$ is the log$_{10}$ value of the copies (Supplementary Fig. S3, available at JXB online). The copies were calculated using the equation: copies µl$^{-1}$ = 6.02 × 10$^{18}$ × concentration of DNA (ng µl$^{-1}$) × (DNA length/660). For the pathway genes, the -2ΔΔCt method was applied to calculate the relative expression level, calibrated using the geometric average (Huggett et al., 2005; Vandesompele et al., 2002) of the S. miltiorrhiza actin, Ub, and EFL genes as the internal reference and normalized with the control samples. Each genotype of transgenic hairy roots was assayed with three independent biological replicates.

Isolation and characterization of the SmMDC promoter region

The 5′-upstream regions of SmMDC (GenBank accession no. JX113690) were cloned using a PCR-based 5′-DNA walking strategy according to the protocol provided by the GenomeWalker Universal Kit (Clontech, USA). Genomic DNA was separately digested with DraI, EcoRV, PvuI, and Smal, and the digestion products were then linked with adaptors, making four genomic libraries. Two rounds of PCR were performed using adaptor primers (AP1 and AP2) and gene-specific primers (GP1 and GP2P), which were designed according to the coding sequence of the SmMDC genes (Supplementary Table S1). PCR products were cloned into pMD19-T easy vector (TaKaRa) and sequenced. PlantCare (Lescot et al., 2002, Last accessed 27 August 2015), PlantPan (Chang et al., 2008, Last accessed 27 August 2015), and NSITE-PL (Softberry Inc.; Solovyev et al., 2010, Last accessed 27 August 2015) were employed to predict the characteristics of the promoters.

Agrobacterium-mediated transient expression in the dual-luciferase assay

A transient expression assay was carried out using transgenic danshen cells according to the previously described methods (Cazzonelli and Velten, 2006; Song and Wang, 2011) with some modifications. Briefly, a single colony of an individual A. tumefaciens strain was picked and cultured in YEB liquid medium containing kanamycin (80 mg l$^{-1}$) to a final OD$_{600}$ of 0.6 and centrifuged to obtain the cell pellet, which was resuspended in MS liquid medium with 100 µM of acetosyringone and then transferred into tubes containing 0.1 g of fresh danshen cells. A. tumefaciens with p35S::Rluc was supplemented for co-transformation at a ratio of 1:50 for calibrating the reading of firefly luciferase. After cultivation at 25 °C and 120 rpm in a horizontal shaker for 48 h, plant cells were collected for the luciferase assay using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega). In view of the differences in cell growth, co-transformation with p35S::Luc and p35S::Rluc was adopted for normalization. The relative activity of the promoter fragment was expressed as the ratio of the reading of firefly luciferase to that of Renilla luciferase after normalization. Each assay was performed simultaneously in triplicate.

Quantification and identification of salvianolic acids and tanshinones

To extract salvianolic acids, approximately 50 mg of lyophilized hairy root powder was weighed and soaked in 5 ml of 75% methanol with 0.1% (v/v) formic acid under sonication for 40 min. The mixture was centrifuged at 12 000 rpm for 10 min to obtain the supernatant, which was recovered and filtered through 0.2 µm membrane for analysis (Hu et al., 2005; Liu et al., 2007). Tanshinones were extracted in the same way except that the extraction solvent was replaced with pure methanol and the supernatant was condensed to 1 ml before analysis.

Reference standards of RA, SAB, cryptotanshinone (CT), tanshinone I (TA-I), and TA-IIA (Shanghai R&D Center for Standardization of Chinese Medicines, China) were used to determine the concentration. The sum of RA and SAB was taken as total salvianolic acids (TSA) and of CT, TA-I and TA-IIA as total tanshinones (TTA). LC-MS was also employed to identify the other diterpenoids.

It should be noted that all the transgenic hairy roots were used to analyse the biomass and salvianolic acid. Only the hairy roots that grew well [eight C1-, nine C1/R-, six R-, and seven control (P-) hairy roots] were chosen for flavonoid, anthocyanin, and tanshinone quantification.

Extraction and determination of total flavonoids and anthocyanins

Flavonoids and anthocyanins were extracted and determined using photometric methods as described in the literature (Mehrtens et al., 2005; Zhang and Wang, 2009; Zhang et al., 2010) with minor modifications. Briefly, 1 ml of acidic methanol (1% HCl, w/v) was added into 50 mg of lyophilized hairy root powder and incubated for 18 h at room temperature with moderate shaking. After centrifugation of the mixture at 15 400g for 5 min at room temperature, 400 µl of the supernatant was transferred into a new tube and 600 µl of acidic methanol was added. For flavonoids, absorbance at 510 nm was monitored after successively supplementing the extract with 5% NaNO$_3$, 10% AlCl$_3$, and 4% NaOH. Concentration was calculated based on the calibration standard curve of (–)-epicatechin ($y=1.19x-0.0817$, $R^2=0.9907$, 0.1–0.3 mg ml$^{-1}$) and expressed as µg (–)-epicatechin equivalents (EE) g$^{-1}$ of sample. For anthocyanins, absorption was monitored at 530 and 657 nm. Quantification of anthocyanins was done using the following equation: $Q_{Anthocyanins} = (A_{530} - 0.25\times A_{657})\times M^{-1}$, where $Q_{Anthocyanins}$ is the amount of anthocyanins, $A_{530}$ and $A_{657}$ are the absorption readings at the individual wavelengths and $M$ is the weight (g) of the material used for extraction (Mehrtens et al., 2005).

HPLC-PDA-MS$^\text{SM}$ conditions

HPLC analysis was carried out with a Beckman Coulter$^\text{TM}$ ODS column (250 × 4.6 × 5 mm). For salvianolic acid analysis, a gradient elution of solvent A (acetonitrile) and solvent B (water:acetonitrile:formic acid=90:10:0.4) at a flow rate of 1.0 ml min$^{-1}$ was used as follows: 0–40 min, A 0–30%, B 100–70% was applied. The injection volume was 10 µl and the detection wavelength was 280 nm at 25 °C. For the tanshinone assay, the program was 0–25 min, solvent A (acetonitrile) 60%, solvent B (water), 40%; 25–26 min, solvent A, 60–80%, solvent B, 40–20%; 26–35 min, solvent A 80%, solvent B 20%. The injection volume was 10 µl and the detection wavelength was 270 nm.

MS analysis was performed using a LCQ ion trap instrument (Thermo Finnigan, San Jose, USA) equipped with an Xcalibur$^\text{TM}$ workstation. The positive-ion mode for MS analyses was selected, working under the following conditions: capillary voltage 19 V, spray voltage 5.0 kV, capillary temperature 300 °C, sheath gas flow rate at 40 (arbitrary units), auxiliary gas flow rate at 20 (arbitrary units), and tube lens offset 40 V. The full scan mass spectra were recorded in the range of m/z 150–800. The isolation width of precursor ions was 1.0 Th. The HPLC/MS data were acquired and processed using the Finnigan Xcalibur 1.3 software provided by the manufacturer.

Results

Morphology and development of transgenic danshen hairy roots

Antibiotic-resistant hairy roots were cultured in liquid medium to obtain enough material for PCR selection from
which 23, 16, 17, and 16 independent lines of C1-, C1/R-, R-, and P-hairy roots (the control) were obtained. After subculture for several generations, independent of whether they were cultured in liquid or solid medium, hairy roots with the same genotype gradually exhibited specific morphological and developmental traits. Representative images of these hairy roots are shown in Fig. 2A–C. Compared with the control (P7 and P16), C1-hairy roots were longer but with fewer lateral hairy roots, as were the C1/R-modified ones: R-hairy roots were thicker than the control, especially when they were cultured in solid medium (Fig. 2C). It is important to note that hairy roots with C1 or C1/R were prone to dedifferentiation to form a callus in contrast to R-engineered ones, as indicated in Fig. 2B. The biomass of C1- and C1/R-hairy roots was significantly lower (23 and 13%, respectively) compared with the control. Propagation of R-hairy roots was slower,

![Fig. 2. Morphology and biomass of transgenic hairy roots of S. miltiorrhiza. (A, B) Typical morphology of hairy roots cultured in liquid MSOH medium for 2 weeks. Arrows indicate the callus. (C) Hairy roots cultured in solid MSOH medium for 2 months (photographed through the bottom of the flask). (D) Biomass of hairy roots cultured in liquid MSOH medium for 3 weeks. The data represent means±SE of 23, 16, 17, and 16 independent biological replicates for C1-, C1/R-, R-, and control hairy roots. The significance of difference between the control and transgenic hairy roots was calculated using Student’s t-test. The same or different lowercases above the columns indicate no significant difference (P>0.01) or significant difference among the samples (P<0.01), respectively. (This figure is available in colour at JXB online.)](https://academic.oup.com/jxb/article-abstract/66/22/7211/2893282)
but they achieved the same biomass as control roots after 3 weeks.

Absolute expression levels of the C1 and R genes in transgenic danshen hairy roots

Absolute expression levels of C1 and R were assayed in individual transgenic hairy roots that were growing well. These hairy roots were arranged in descending order based on the dosage of C1 or R (Fig. 3). The expression level of C1 in C1-8 was the highest, followed by C1-11 and C1-6 (Fig. 3A); that of R in R-21 hairy root was the highest, followed by R-16 and R-13 (Fig. 3B). As for C1/R-hairy roots, only four C1/R-hairy roots grew well and were kept for the assay, in which C1/R-18 showed the highest C1 and R expression level, followed by C1/R-12, C1/R-2 and C1/R-6 (Fig. 3C). The expression level of C1 and R was consistent with each other in C1/R-hairy roots, possibly due to C1 and R having been assembled together into the same vector for co-transformation.

Production of salvianolic acids

Being the dominant phenylpropanoid metabolites and bioactive ingredient in danshen, salvianolic acids were firstly measured in all the transgenic hairy roots using SAB and RA as the representative compounds. Unexpectedly, all the hairy roots modified with C1 or C1 coupled with R produced less RA and SAB than the control, as shown in the typical HPLC profiles (Fig. 4A). The production of TSA in C1- and C1/R-hairy roots was 52.2 ± 4.2 mg g⁻¹ of DW and 58.6 ± 5.6 mg g⁻¹ of DW, 37.3 and 42.5% of the control, respectively (Table 1, Fig. 5A). With regard to individual hairy roots, C1-22 produced the highest amount of TSA, which was 106.1 mg g⁻¹ of DW and C1-8 produced the lowest with 20.4 mg g⁻¹ of DW of TSA, which was exactly the opposite of the expression level of C1 in these two hairy root lines (Fig. 3A). Among C1/R-hairy roots, C1/R-21 showed the highest TSA-producing capability, yielding 115.8 mg g⁻¹ of DW of TSA, 82.7% of the control. R-modified hairy roots accumulated 68.1 ± 2.3 mg g⁻¹ of DW of RA and 70.6 ± 3.8 mg g⁻¹ of DW of SAB, nearly equal to the control (95.3 and 103.0%, respectively) (Table 1, Fig. 5A). R-13 and R-20 were the top two highest TSA-producing R-hairy roots but not the highest R-expressing ones (Fig. 3B). Taken together, it appeared that the heterologous expression of C1 alone or C1 coupled with R inhibited the biosynthesis of salvianolic acids, although R itself had little effect on the accumulation of salvianolic acids in transgenic danshen hairy roots.

Production of total flavonoids and anthocyanins

Belonging to the same class of phenylpropanoid metabolites as salvianolic acids, flavonoids and anthocyanins were quantified using a photometric method. The average production of total flavonoids in C1- and C1/R-hairy roots was 3.4 ± 0.5 mg of EE g⁻¹ of DW and 3.3 ± 0.7 mg of EE g⁻¹ of DW, respectively (Fig. 5B), which was less than that in the control of 3.6 ± 0.4 mg of EE g⁻¹ of DW but without being significantly different (P>0.01). C1-21 accumulated the highest amount of flavonoids (Supplementary Fig. S5A, available at JXB online). Flavonoids in R-modified hairy roots were 5.1 ± 0.8 mg of EE g⁻¹ of DW; R-13 yielded the highest amount of flavonoids at 6.96 mg of EE g⁻¹ of DW (Supplementary Fig. S5B), which was higher than that in the control but without being significantly different (P>0.01) (Table 1, Fig. 5B). Regarding the anthocyanins, C1- and C1/R-hairy roots produced 6.85 ± 0.65 and 7.3 ± 0.8 absorbance units (AU) g⁻¹, which was 1.7- and 1.8-fold higher than the control, respectively, and C1/R-2 showed the highest anthocyanin concentration (Supplementary Fig. S5C). R-hairy roots produced nearly equal amounts of anthocyanins as the control (Fig. 5B). These results suggested that expression of maize R had a positive effect on the biosynthesis of flavonoids, and that expression of C1 alone or C1 coupled with R was beneficial for the accumulation of anthocyanins in transgenic danshen hairy roots.

Production of tanshinones

As the other dominant therapeutic compounds of danshen, tanshinones were monitored with HPLC and LC-MS. Nine compounds were detected and seven were identified as 15,16-dihydrotanshinone I, 1,2-didehydrodimiltirone, methyltanshinonate, CT, TA-I, 1,2-dihydrotanshinone I and TA-IIA (Table 2). Interestingly, all nine compounds were synthesized at higher levels in C1- and C1/R-hairy roots than in R-hairy roots and the control (Fig. 4B). The average production of CT, TA-I, and TA-IIA was 0.15 ± 0.04, 0.91 ± 0.09, and 1.23 ± 0.13 mg g⁻¹ of DW in C1-hairy roots, and 0.17 ± 0.05, 0.75 ± 0.07 and 0.79 ± 0.10 mg g⁻¹ of DW in C1/R-hairy roots, respectively (Table 3, Fig. 6). With regard to the individual hairy root line, the maximum production of tanshinones was detected in C1-6 hairy roots and was 3.59 mg g⁻¹ of DW of TTA, 3.42-fold higher than the control (Fig. 7A). C1-6 also possessed the second highest C1 dosage among C1-hairy roots (Fig. 7A). For C1/R-hairy roots, C1/R-5 was the most productive line, producing 2.85 mg g⁻¹ of DW of TTA (Fig. 7C). The total tanshinones in R-hairy roots ranged from 0.71 in R-21 to 1.74 mg g⁻¹ of DW in R-2, with an average production of TTA of 1.05 ± 0.15 mg g⁻¹, which was almost equal to the control (Table 3, Figs 4B, 6, and 7B). These results demonstrated that expression of maize C1 improved the accumulation of tanshinones, while R itself had almost no impact on danshen hairy roots.

Relative expression levels of phenylpropanoid pathway genes

Transcription levels of genes involved in the biosynthesis of salvianolic acids (Hedden and Thomas, 2012) and other phenylpropanoids were explored in the transgenic hairy roots. Three lines of each genotype of hairy roots were chosen as biological replicates; these were C1-6, C1-8, and C1-9 for C1-hairy roots; C1/R-2, C1/R-6, and C1/R-12 for C1/R-hairy roots; and R-13, R-16, and R-20 for R-hairy roots. P7, P15, and P16 were used as the controls for normalization. As shown
ZmC1 increased the tanshinone level in danshen hairy roots

in Fig. 1, among the five general phenylpropanoid pathway genes, *SmPAL1* was almost unchanged in all the transgenic hairy roots, *SmPAL2* was decreased in C1- and C1/R-hairy roots but upregulated in R-hairy roots, and *SmC4H, Sm4CL1*, and *Sm4CL2* were upregulated in all three transgenic hairy roots and all of them showed the lowest expression level in R-hairy roots. For the two tyrosine pathway genes, *SmTAT* was inhibited in C1- and C1/R-hairy roots but was unchanged in R-hairy roots, while *SmHPPR* was repressed in C1- and R-hairy roots and unchanged in C1/R-hairy roots.

Fig. 3. Absolute expression levels of C1 and R with the production of salvianolic acids in individual transgenic hairy roots of *S. miltiorrhiza*. (A) C1-hairy roots. (B) R-hairy roots. (C) C1/R-hairy roots. All the transgenic hairy root lines were arranged in descending order based on the number of copies of C1 or R. Data for the copies represent means±SE of three replicates. Data for salvianolic acids in individual samples are from one batch of cultured hairy roots (n=1). Columns with a cross above indicate that the sample was not used to perform qRT-PCR. (This figure is available in colour at *JXB* online.)
Regarding the genes responsible for the RA biosynthetic pathway (*SmRAS, SmCPR, and SmCYP98A14*), flavonoid biosynthetic genes (*SmCHS and SmF3′H*), and lignin biosynthetic genes (*SmCCR, SmC3H-1, SmC3H-2, SmCOMT, SmCAD, and SmGT*), all were upregulated in C1-hairy roots, and SmCAD showed the highest level at 7.6±3.0, indicating that expression of maize *Cl* regulated most phenylpropanoid genes. In particular, it distinctly activated the transcription of *SmC4H* and *SmCAD* and suppressed the expression of *SmTAT* and *SmHPPR*. In C1/R-hairy roots, besides *SmC4H, Sm4CL1*, and *Sm4CL2, SmCPR, SmCHS, and SmCCR* were all upregulated, and the expression level of *SmC4H, Sm4CL1, Sm4CL2, and SmCHS* was higher than that in C1-hairy roots, but the transcript levels of *SmRAS, SmCYP98A14, SmF3′H, SmC3H-1, SmC3H-2, SmCOMT, SmCAD, and SmGT* were similar to the control, suggesting that co-expression of R boosted the activation that C1 confers to certain genes but not to all genes. In R-hairy roots,
Table 1. Production of phenylpropanoids in transgenic hairy roots of S. miltiorrhiza

Data for salvinolic acids from C1-, C1/R-, R-, and the control hairy roots represent means±SE of 23, 16, 17, and 16 independent biological replicates, while those of flavonoids and anthocyanins represent means±SE of eight, nine, six, and seven independent biological replicates. Numbers in the parentheses indicate the percentage of phenylpropanoids in transgenic hairy roots compared with the control. The sum of RA and SAB was taken as TSA.

<table>
<thead>
<tr>
<th>Hairy roots</th>
<th>Production of salvianolic acids (mg g⁻¹ of DW)</th>
<th>Total flavonoids (mg of EE g⁻¹ of DW)</th>
<th>Total anthocyanins (Q_{Anthocyanins}=(A_{650}−0.25×A_{630})×M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RA (mg g⁻¹ of DW)</td>
<td>SAB (mg g⁻¹ of DW)</td>
<td>TSA (mg of EE g⁻¹ of DW)</td>
</tr>
<tr>
<td>C1</td>
<td>28.4 ± 1.7 (39.7%)</td>
<td>23.8 ± 2.7 (34.7%)</td>
<td>52.2 ± 4.2 (37.3%)</td>
</tr>
<tr>
<td>C1/R</td>
<td>32.0 ± 2.9 (44.8%)</td>
<td>26.6 ± 2.8 (38.8%)</td>
<td>58.6 ± 5.6 (42.5%)</td>
</tr>
<tr>
<td>R</td>
<td>68.1 ± 2.3 (95.3%)</td>
<td>70.6 ± 3.8 (103.0%)</td>
<td>138.7 ± 3.9 (99.0%)</td>
</tr>
<tr>
<td>Control</td>
<td>71.5 ± 2.9 (100.0%)</td>
<td>68.6 ± 2.8 (100.0%)</td>
<td>140.1 ± 4.1 (100.0%)</td>
</tr>
</tbody>
</table>

Fig. 5. Production of phenylpropanoids in transgenic hairy roots of S. miltiorrhiza. (A) Production of salvianolic acids. (B) Production of total flavonoids and anthocyanins. Data for salvianolic acids for C1-, C1/R-, R-, and the control hairy roots represent means±SE of 23, 16, 17, and 16 independent biological replicates, while data for flavonoids and anthocyanins represent means±SE of eight, nine, six, and seven independent biological replicates. One-way ANOVA and Student’s t-test were performed to identify significant differences. The same or different lowercases above the columns indicate no significant difference (P>0.01) or significant difference among the samples within a single compound (P<0.01), respectively. (This figure is available in colour at JXB online.)
besides the five general phenylpropanoid pathway genes, the transcripts of SmCPR, SmCYP98A14, SmCHS, SmC3H-2, SmCOMT, and SmCAD were increased, those of SmRAS, SmCCR, SmC3H-1, and SmGT were decreased, and SmF3’H was unchanged, indicating that R could also interfere with the transcription of some phenylpropanoid genes.

### Table 2. Characterization of diterpenoids identified from transgenic hairy roots of Salvia miltiorrhiza

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>t_{R} (min)</th>
<th>Assigned identity</th>
<th>Molecular ions m/z (Da)</th>
<th>Fragment ions m/z (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1’</td>
<td>8.41</td>
<td>Unknown</td>
<td>487.50</td>
<td>469.97, 425.10, 371.07</td>
</tr>
<tr>
<td>2’</td>
<td>13.44</td>
<td>15,16-Dihydrotanshinone I</td>
<td>278.90</td>
<td>260.73, 232.72</td>
</tr>
<tr>
<td>3’</td>
<td>14.78</td>
<td>1,2-Didehydromiltirone</td>
<td>280.91</td>
<td>262.64, 234.78, 220.76</td>
</tr>
<tr>
<td>4’</td>
<td>16.16</td>
<td>Methyltanshinonate</td>
<td>338.90</td>
<td>278.56, 260.67</td>
</tr>
<tr>
<td>5’</td>
<td>19.57</td>
<td>Cryptotanshinone</td>
<td>296.90</td>
<td>278.70, 263.73, 250.80, 235.75, 222.78, 208.76</td>
</tr>
<tr>
<td>6’</td>
<td>21.96</td>
<td>Tanshinone I</td>
<td>276.90</td>
<td>248.76, 220.74, 192.78</td>
</tr>
<tr>
<td>7’</td>
<td>25.60</td>
<td>1,2-Dihydrotanshinone I</td>
<td>278.90</td>
<td>260.70, 232.73</td>
</tr>
<tr>
<td>8’</td>
<td>30.41</td>
<td>Tanshinone II A</td>
<td>295.10</td>
<td>276.71, 248.81, 233.74, 220.74</td>
</tr>
<tr>
<td>9’</td>
<td>37.28</td>
<td>Unknown</td>
<td>283.20</td>
<td>266.98</td>
</tr>
</tbody>
</table>

### Table 3. Production of tanshinones in transgenic hairy roots of S. miltiorrhiza

The expression levels of 22 terpenoid genes associated with the MVA pathway and the MEP pathway were assayed using the same samples as those for the phenylpropanoid pathway genes. As shown in Fig. 1, the relative expression levels of

### Relative expression levels of terpenoid biosynthetic genes

The expression levels of 22 terpenoid genes associated with the MVA pathway and the MEP pathway were assayed using the same samples as those for the phenylpropanoid pathway genes. As shown in Fig. 1, the relative expression levels of
ZmC1 increased the tanshinone level in danshen hairy roots. All the investigated genes were upregulated in C1-hairy roots, including the upstream genes for IPP and the downstream genes for individual terpenoid derivatives, among which SmMDC showed the highest level at 26.7 ± 7.9, followed by SmPMK at 12.1 ± 4.3, demonstrating that expression of C1 improved the transcription of terpenoid genes, especially...
SmMDC and SmPMK. In C1/R-hairy roots, 16 out of 22 genes were upregulated of which 14 were lower than those in C1-hairy roots, but the other two MEP pathway genes, SmDXS1 and SmCPS, were higher than in C1-hairy roots; moreover, SmPMK was downregulated and SmAACT, SmHMGS, SmMK, SmMCT, SmIPPI, and SmFPSS were at about the same level as those in the control, which suggested that, instead of synergistically acting with C1, co-expression of R reduced the stimulation effect of C1 on most terpenoid genes. In R-modified hairy roots, for the MVA pathway, the transcripts of SmHMGR1, SmHMGR, SmHMGR3, SmMK, and SmMDC were increased, the transcript level of SmFPSS was at the same level as the control, and the transcripts of SmAACT, SmHMGS, and SmPMK were decreased; for the MEP pathway, SmDXS1, SmMCT, SmHDS, and SmCPS5 were upregulated, SmCMK, SmMDS, and SmHDR were unaffected, and SmDXS2, SmDXR, SmIPPI, SmGGPPS, SmCPS, and SmKSL were downregulated, which indicated that ectopic expression of R itself also had a certain regulatory effect on some terpenoid genes.

**Isolation and characterization of the SmMDC promoter**

Since SmMDC was the most significantly upregulated gene, cloning of its promoter region was undertaken for further study. A 2042 bp DNA fragment (GenBank accessionno. JX113690) upstream of the translation initiation codon was obtained with a typical TATA box present at –30 bp upstream of the transcription start site (Supplementary Fig. S4). Prediction with PlantCARE, PlantPan, and NSITE-PL showed that a number of potential cis-acting elements corresponding to different regulatory signals were located in this region (Supplementary Table S2 and Supplementary Fig. S4). Available at JXB online, containing motifs responsible for endosperm-, pollen-, and root-specific expression such as GCN4_motif, Skn-1_motif and Pollen1, elements responding to defence and stress signals such as ARE, W-box, GT-1, HSE, LTR, WUN-motif, and TC-rich repeats, and elements involved in hormone signalling such as abscisic acid, auxin, ethylene, gibberellins, and salicylic acid, as well as some light-responsive ones. The presence of such cis-elements indicated that SmMDC might be important in responding to endogenous and exogenous signals in *S. miltiorrhiza*.

Interestingly, there were a number of transcription factor binding sites belonging to typical phenylpropanoid biosynthetic genes at the SmMDC promoter region. The core sequences of TCTTACCTACCACC [-1267(+) to –1253(+)] and GTTAGGGT [-1142(+) to –1135(+)], were respectively identical to one of the putative cis-elements, boxes P, A, L, and AC-II, found in *PcPAL, DcPAL, AtchsA, AtC4H, AtF3H, AtACS1,* and *ZmCOMT,* of which Box P in the PAL, CHS, CHI, DFR, CL, Bz1, and Myb305 promoters were binding sites of plant MYB transcription factors. In addition, a number of C1-binding sites including C1 motif, P (B-Myb), and MYB PZm, and especially C1 PBS/P, a highly conserved C1 recognition sequence of ‘ACCTACCAcCC’ found in the maize *al* gene, were also located in the SmMDC promoter region (Supplementary Table S2, Supplementary Fig. S4). The special feature shared by the SmMDC promoter with the phenylpropanoid biosynthetic genes gave a hint that the biosynthesis of terpenoids might be associated with phenylpropanoid metabolism in some way.

**Luciferase-based transient expression assay in C1-modified cells**

To explore the possible interaction between C1 and the SmMDC promoter, three luciferase-based plant expression vectors with truncated SmMDC promoter fragments were constructed, taking into account both the predicted putative transcription start site, TATA box, and CAAT box, and the distribution of C1 binding sites, yielding vectors pF1::Luc, p5’F1::Luc, and pF2::Luc, where F1 represented the –1316/+87 fragment containing seven C1 motifs, one C1 PBS/P, and two P (B-Myb) [the MYB PZm and one P (B-Myb) was covered by C1 PBS/P and was not counted]; 5’F1 was the –1316/–548 fragment with four C1 motifs, one C1 PBS/P, and one P (B-Myb); and F2 was the –532/+87 fragment with three C1 motifs and one P (B-Myb) (Supplementary Table S2, Supplementary Fig. S4). As shown in Fig. 8, the transcriptional activity of F1 and F2 were nearly equal, while that of 5’F1 was only about 36% of F1 in the control transient transformed cells, which indicated that the –532/+87 fragment could function as a primary promoter and the deletion of the –1316/–532

![Fig. 8.](https://academic.oup.com/jxb/article-abstract/66/22/7211/2893282)
Table 4. Correlations between C1 copies and the production of secondary metabolites in C1-hairy roots of S. miltiorrhiza

Correlation coefficients were from Pearson correlation analysis carried out with SPSS19.0 using 15 independent C1-hairy root lines for salvianolic acids and eight hairy root lines for flavonoids, anthocyanins and tanshinones.

<table>
<thead>
<tr>
<th>Items</th>
<th>C1 copies</th>
<th>RA</th>
<th>SAB</th>
<th>TSA</th>
<th>Flavonoids</th>
<th>Anthocyanins</th>
<th>CT</th>
<th>TA-I</th>
<th>TA-IIA</th>
<th>TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 copies</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>-0.875**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAB</td>
<td>-0.857**</td>
<td>0.828**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA</td>
<td>-0.901**</td>
<td>0.933**</td>
<td>0.974**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-0.364</td>
<td>0.408</td>
<td>0.489</td>
<td>0.471</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>0.918**</td>
<td>-0.851**</td>
<td>-0.942**</td>
<td>-0.932**</td>
<td>-0.526</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>0.589</td>
<td>-0.491</td>
<td>-0.599</td>
<td>-0.573</td>
<td>-0.531</td>
<td>0.810*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA-I</td>
<td>0.713*</td>
<td>-0.800*</td>
<td>-0.797*</td>
<td>-0.819*</td>
<td>-0.291</td>
<td>0.853**</td>
<td>0.703</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA-IIA</td>
<td>0.795*</td>
<td>-0.657</td>
<td>-0.716*</td>
<td>-0.712*</td>
<td>-0.361</td>
<td>0.882**</td>
<td>0.920*</td>
<td>0.722*</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TTA</td>
<td>0.790*</td>
<td>-0.732*</td>
<td>-0.781*</td>
<td>-0.782*</td>
<td>-0.395</td>
<td>0.928**</td>
<td>0.927**</td>
<td>0.878**</td>
<td>0.961**</td>
<td>1</td>
</tr>
</tbody>
</table>

*Correlation is significant at the P<0.05 level (two-tailed).
**Correlation is significant at the P<0.01 level (two-tailed).

region did not affect the basic modulating activity; furthermore, the −1316−548 fragment could act as an independent promoter possibly attributed to one of the predicted transcription start sites (Supplementary Fig. S4). In C1-6 cells, the relative activity of F1::Luc, 5′F1::Luc, and F2::Luc was as high as 1.6-, 7.4-, and 1.7-fold that of the corresponding constructs in P16 cells, demonstrating that maize C1 activated the truncated SmMDC promoter fragments. It also showed that 5′F1 was stronger than CaMV 35S promoter when C1 was present.

Discussion

The ZmC1-modulated increase of tanshinones and decrease of salvianolic acids results from engineering of both pathway genes

Maize C1 and R were applied to manipulate the biosynthesis of anthocyanins and flavonoids by co-expressing them in tomato fruits and maize cells (Grotewold et al., 1998; Bovy et al., 2002). Due to the possible activation to a more diverse set of promoters (Sainz et al., 1997), maize C1 and R were introduced into danshen hairy roots with the aim of accelerating the biosynthesis of salvianolic acids. Unexpectedly, the production of salvianolic acids was reduced but the accumulation of tanshinones was significantly improved in C1- and C1/R-hairy roots, which prompted us to explore the putative regulation mechanism.

The synthesis of salvianolic acids requires two necessary precursors, 4-coumaroyl-CoA and 4-HPLA, respectively from the general phenylpropanoid and tyrosine pathways (Fig. 1), which means that both pathways are essential for the formation of salvianolic acids. As shown by qRT-PCR (Fig. 1), three phenylpropanoid genes were clearly inhibited in C1-hairy roots and two of them, SmPAL2 and SmTAT, were also suppressed in C1/R-hairy roots. Since SmPAL1 is homologous to SmPAL2 and its expression was not affected in those hairy roots, this suggests that SmTAT, the entry-point gene of the tyrosine pathway, plays a crucial role in the ZmC1-modulated decrease of salvianolic acids. It is likely that the diminished transcripts of SmTAT and consequently the enzyme activity limited the conversion of tyrosine to 4-HPPA, reduced the supply of 4-HPLA, and finally resulted in a decrease of salvianolic acids. However, SmPAL1 was almost unaffected, while the transcription of the other general phenylpropanoid genes and the RA branch pathway genes was increased. SmHPPR might also contribute to the decrease of salvianolic acids in C1-hairy roots. Yet, taking into account its transcription pattern and the production of salvianolic acids in C1/R- and R-hairy roots (Figs 1 and 5), it appears that SmHPPR is less important than SmTAT. In the past, the importance of SmTAT on the formation of RA and SAB was also verified by overexpression of SmTAT and SmHPPR in danshen hairy roots where the production of RA and SAB was increased 16.1 and 18.8 times over that of the control (Xiao et al., 2011).

Furthermore, as an anthocyanin-regulating transcription factor, it was reasonable to expect that expression of C1 or C1 with R would accelerate the synthesis of anthocyanins in C1- and C1/R-hairy roots. The C1-stimulated upregulation of Sm4CL, Sm4CH, SmCHS, SmF3′H, and possibly some other unknown genes responsible for the biosynthesis of anthocyanins pushed the carbon flux into anthocyanins in spite of the slightly changed expression of SmPAL. As a result, the metabolic flow to salvianolic acids was reduced, since salvianolic acids share the same 4-coumaryl-CoA precursor as anthocyanins (Fig. 1), which, combined with the reduced supply of 4-HPLA, further decreased the biosynthesis of salvianolic acids.

As the partner of C1 responsible for anthocyanins (Grotewold et al., 1998; Bovy et al., 2002), it was possible that R cooperated primarily with C1 in regulating certain anthocyanin-related genes in view of the higher level of Sm4CL2 and SmCHS as well as the higher accumulation of anthocyanins in C1/R-hairy roots than in C1-hairy roots. Meanwhile, R itself showed little effect on the accumulation of phenylpropanoids except for flavonoids (Table 1, Fig. 5B). The increase of flavonoids in R-hairy roots was possibly the result of the notable upregulation of SmPAL2 transcripts and possibly some other flavonoid-related genes (Fig. 1). Why co-expression of R with
C1 reduced the transcripts of most other genes (especially SmC3H-I and SmCAD) needs to be elucidated in the future. In contrast to salvianolic acids, the production of tanshinones was significantly increased in C1-hairy roots. All nine diterpenoids detected were increased (Fig. 4B), and the average production of TTA was 2.2-fold higher than the control (Table 3). As described above, the transcripts of all the terpenoid pathway genes in C1-hairy roots were upregulated including the upstream MVA pathway and MEP pathway genes for IPP and the downstream genes for various terpenoid derivatives, and most of them showed higher levels in C1-hairy roots than in the others (Fig. 1). Such comprehensive and high upregulation of pathway genes therefore led to the subsequent improvement of tanshinone production.

C1/R-hairy roots also accumulated more diterpenoids, and the average production of TTA was 1.63-fold higher than that of the control (Table 3). Compared with their behaviour in C1-hairy roots, the terpenoid pathway genes in C1/R-hairy roots showed a large difference in that the transcripts of two MEP pathway genes, SmDXS1 and SmCPS, rose significantly, and the transcripts of some MVA pathway genes, SmAACT, SmHMGS, SmHMGR, SmMK, SmPMK, and SmMDC, and SmFPPS were decreased drastically. The high expression level of SmDXS1 and SmCPS was possibly the main attribution to the improvement of tanshinones in C1/R-hairy roots in contrast to the control. However, the decreased transcription of SmAACT, SmHMGS, SmHMGR, SmMK, SmPMK, and SmMDC reduced the production of tanshinones less than in C1-hairy roots. As a downstream gene involved in terpenoid derivatives biosynthesis rather than diterpenoid biosynthesis, the decrease of SmFPPS might help to drive IPP to diterpenoids.

Elicitor-induced tanshinones are generally believed to be derived mainly from the MEP pathway (Ge and Wu, 2005; Xu et al., 2010; Zhao et al., 2010; Gao et al., 2014). The current study showed that the heterologous transcription factor-stimulated tanshinone accumulation was also derived primarily from the MEP pathway. Furthermore, the highly expressed MVA pathway genes, in particular SmPMK and SmMDC, and consequently elevated tanshinone production in C1-engineered hairy roots highlighted the importance of MVA pathway in tanshinone biosynthesis.

Transcription factors could channel the metabolic flux by simultaneously regulating the transcription of related biosynthetic genes, making them promising tools for manipulating secondary metabolism (Gantet and Memelink, 2002; Broun, 2004; Wurtzel and Grotewold, 2006; Grotewold, 2008). Compared with the intensive study and application of MYB transcription factors in modulating the phenylpropanoid metabolism (Bovy et al., 2002; Stracke et al., 2007; Luo et al., 2008; Palapal et al., 2009; Feng et al., 2010; Hichri et al., 2011; Ma et al., 2013), knowledge about the MYB effect on terpenoids is rather limited. For instance, heterologous expression of grapevne VvMYB5b in tomato increased the carotenoid content (Mahjoub et al., 2009). Ectopic expression of the PriMYB14 gene in P. glauca led to the accumulation of sesquiterpene in conifers (Bedon et al., 2010). The results of the current study provide more evidence that the generally accepted phenylpropanoid MYB transcription factor could affect the terpenoid pathway, at least ectopically.

The ZmC1-mediated decrease of salvianolic acids and increase of tanshinones is significantly negative correlated, indicating crosstalk between these metabolites

Correlation analysis with the data of individual C1-hairy roots as shown in Fig. 3A and Fig. 7A showed that C1 copies were significantly negatively correlated with RA, SAB, and TSA at the P<0.01 level and significantly positively correlated with TA-I, TA-IIA, and TTA at the P<0.05 level (Table 4), which indicated that the decrease of salvianolic acids and increase of tanshinones was really attributed to the expression of C1. As C1 is a MYB transcription factor responsible for phenylpropanoid biosynthesis, it was interesting to elucidate how C1 activated the accumulation of tanshinones but inhibited the biosynthesis of salvianolic acids.

Maize C1 activated the al promoter by direct sequence-specific binding and demonstrated a relatively broad DNA-binding specificity with the highest affinities shown towards a similar plant Myb domain protein consensus binding site (A/C|A|C) (Sainz et al., 1997). In view of this, the upstream region of SmMDC was cloned and submitted for promoter prediction. There was a highly covered C1-binding site, C1 PBS/P (ACCTACCCACCC) at −1263(+) to −1253(+) as well as other C1 recognition sequences including P (B-Myb), C1 motif, and MYB PZm in the SmMDC promoter (Supplementary Table S2). A further transient expression assay with a truncated SmMDC promoter revealed that the −1316−548 fragment containing four C1 motifs, one C1 PBS/P, and one P (B-Myb) showed stronger activity than the other fragments without C1 PBS/P (Fig. 7), indicating that the C1 PBS/P should be the dominant element in the C1-activated transcription of SmMDC. However, the C1 motif and P (B-Myb) could also interact with C1, as demonstrated by the F1:Luc and F2:Luc activities in C1-6 cells (Fig. 8). The promoter prediction with two available 5′-untranslated regions (UTRs) of terpenoid genes, SmHMGR1 and SmGGPPS, showed that they both had more than one such C1-binding site, except C1 PBS/P (Supplementary Table S3, available at JXB online). These findings suggested that the comprehensive upregulation of terpenoid genes was due to the presence of C1-binding sites at the 5′-UTR of these genes.

As the co-activator of C1, R relieves C1 from plant-specific inhibition and increases the C1 DNA-binding affinity by interacting with C1, which is essential for C1 to activate the transcription of maize anthocyanin genes (Hernandez et al., 2004). In this study, C1 activated the transcription of most phenylpropanoid genes independently of R except for Sm4CL2 and SmCHS. It is possible that endogenous bHLH transcription factors act as the partner, given that 76 putative bHLH unigene have been annotated from danshen plant (Wenping et al., 2011). The differential expression of these pathway genes probably resulted from the different affinities of C1 binding with cis-regulatory elements distributed in individual genes. It is worth mentioning that co-expression of R also reduced the expression level of most terpenoid genes, especially SmPMK and SmMDC, which may be an interesting point to be explored in the future.
In addition, tanshinones and salvianolic acids are the two major phytoalexins in *S. miltiorrhiza*. As indicated by correlation analysis, the concentration of tanshinones and salvianolic acids showed a strong negative correlation with each other at the *P* < 0.05 level (Table 4), which implied that crosstalk possibly exists between the two compounds in C1-hairy roots.

As described above, *SmTAT* played a crucial role in the C1-modulated decrease of salvianolic acids, and *SmDXS1, SmCPS, SmPMK*, and *SmMDC* were mainly responsible for the increase of tanshinones in C1-hairy roots. It was thought that the crosstalk may have happened at the transcription level of these genes. A study of MEP/terpenoid and shikimate/phenylpropanoid pathways indicated that the carbon flow was redirected between the phenylpropanoid and terpenoid pathways owning to differential gene expression at major metabolic branch points such as *DXS* and *DXR* (Xie et al., 2008), which showed that the metabolic flux could be changed between these two major secondary metabolites by modulating the expression of key genes at the transcriptional level.

The feature of the promoter of *SmMDC* and its behaviour in C1- and C1/R-engineered danshen hairy roots suggested that phenylpropanoid and terpenoid genes shared similar *cis*-regulatory elements. These *cis*-elements appear to contribute to the C1-stimulated crosstalk between the two major secondary metabolites.

During their long evolution period, plants have developed sophisticated mechanisms for controlling and balancing the metabolite flux. *Cis*-regulation is such a mechanism. Studying *cis*-regulatory sequences offers new perspectives for a better understanding of evolution (Stracke et al., 2007). It also offers a better understanding of the transcription factor-mediated shut-off and -on regulatory mechanism. Investigation of characteristics of metabolic pathway genes will be helped by predicting the metabolic flux and utilizing transcription factors, a powerful tool for manipulation and channelling the carbon flow in plants (Broun, 2004; Gantet and Memelink, 2002; Grotewold, 2008; Wurtzel and Grotewold, 2006). The findings of this study help us to understand the connection between the terpenoid and phenylpropanoid metabolism. Elucidation of the metabolic networks could lead to engineering of microbial hosts for the production of these compounds, as has been reported in the literature for other polyphenolic compounds (Xu et al., 2013; Zhao et al., 2015).

It is worth noting that the differences in morphology and development of C1- and C1/R-hairy roots from the control possibly resulted from the variation of endogenous hormones. Like tanshinones, gibberellins belong to abietane-type diterpenoids and abscisic acid is a carotenoid derivative (Milborrow, 2001; Hedden and Thomas, 2012). Whether these hormones participate in the crosstalk between terpenoids and phenylpropanoids is worthy of being investigated in the future.

**Conclusion**

Heterologous expression of maize *CI* significantly improved the accumulation of tanshinones through upregulation of the transcripts of terpenoid biosynthetic genes. *Cis*-acting elements shared by both pathway genes possibly contributed to the C1-stimulated crosstalk between tanshinones and salvianolic acids in C1-hairy roots. To the best of our knowledge, this is the first report about bidirectional regulation of two major bioactive ingredients in *S. miltiorrhiza*.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig S1.** Schematic diagram of constructed plant expression vectors.

**Supplementary Fig S2.** PCR results of selected transgenic hairy roots of *S. miltiorrhiza*.

**Supplementary Fig S3.** Standard curve of Ct value to CI and R copies.

**Supplementary Fig S4.** Sequence of *SmMDC* promoter region.

**Supplementary Fig S5.** Production of flavonoids and anthocyanins in individual transgenic hairy roots.

**Supplementary Table S1.** Primers used in this study.

**Supplementary Table S2.** Putative *cis*-elements in the promoter region of *SmMDC* gene.

**Supplementary Table S3.** Putative *cis*-elements related to MYB binding of 5′-UTR of *HMGR* 1 and *GGPPS*.

**Acknowledgements**

This work was financially supported by the National Natural Science Foundation of China (no. 30973878) and the Innovation Program of Shanghai Municipal Education Commission (no. 14ZZ117). The authors greatly appreciate the help of Professor Matthees A.G. Koffas, Rensselaer Polytechnic Institute (Troy, NY, USA) for his help with editing the manuscript, and Professor Zhibi Hu, Professor Zhengtao Wang, and Mrs Jiyan Zhou, Shanghai University of Traditional Chinese Medicine, for their valuable comments and suggestions.

**References**


