5-Azacytidine Increases Tanshinone Production in Salvia miltiorrhiza Hairy Roots Through Epigenetic Modulation

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Abstract

Recent studies have indicated strong connections between epigenetic modulation and secondary metabolites in plants. It is vital to understand the roles of epigenetics in the production of secondary metabolites. In this study, the DNA demethylation agent 5-azacytidine (5-Az) was used on the hairy roots of the medicinal crop *Salvia miltiorrhiza* to investigate its effect on secondary metabolite production, gene expression, and methylation levels in gDNA and promoter regions. Our results showed that the contents of tanshinones in *S. miltiorrhiza* hairy roots increased by 1.5 to 5 times, and most genes in the MEP pathway showed an upward trend. According to our NGS analysis, the methylation pattern in the copalyl diphosphate synthase (CPS) promoter was altered, and 51 out of 145 cytosines were demethylated during 5-Az treatment. A total of 36 different transcription factors (TFs) were identified in these demethylation sites. Among these TFs, NF-Y and MYB were frequently found in our results. This is the first report to demonstrate a possible mechanism of DNA methylation participating in tanshinone biosynthesis in *S. miltiorrhiza* hairy roots by modulating the CPS promoter and TFs.

Introduction

Epigenetics is an emerging field of biology proposed by Conrad Waddington in 1942 [1]. It investigates how cells can achieve reversible and heritable changes in gene expression without DNA sequence alterations to ensure survival upon environmental fluctuations [2,3]. The underlying mechanisms of epigenetic regulation include DNA methylation, histone modifications, chromatin remodeling, and noncoding RNA (ncRNA)-mediated regulation of gene expression. Among them, DNA methylation transfers the methyl group of S-adenosylmethionine (SAM) to carbon 5 of cytosine at CG, CNG and CNN (where N could be any nucleotide except G) sequences in DNA. The content of 5-methylcytosines is increased through the catalysis of DNA methyltransferase (DNMT) in genomic DNA [4]. In higher plants, several studies have reported that various environmental factors can cause alterations in DNA methylation levels. Changes in methylation patterns in promoter regions might regulate gene expression related to stress resistance [5,6]. For instance, wheat cultivars with different salinity tolerances showed dissimilar methylation patterns and gene expression results when exposed to salinity stress. Demethylation in the promoter region affected the expression of stress resistance genes and improved their survival during environmental fluctuations. This result suggested that DNA methylation might be a key mechanism in the regulation of salinity tolerance in wheat [7,8].

Since environmental stresses have large impacts on plants, some unique strategies, such as stress escape and/or stress tolerance, have been developed to lower the negative effects on their life cycle. Plant secondary metabolites are one of the strategies when plants are exposed to abiotic/biotic stresses, such as water shortages, insect attacks, light, and drought. Secondary metabolites might act as mediators to survive in a tough situation, and their production might be modulated through epigenetic regulation. Recent studies have indicated strong connections between DNA methylation and the production of secondary metabolites, but the relationships have not yet been clearly investigated. Plant

hairy root is a secondary metabolite research platform with the characteristics of rapid growth, easy maintenance, and stable genetic and biochemical characteristics. Plant hairy roots were induced by *Agrobacterium rhizogenes* infection to investigate secondary metabolite production and their biosynthesis pathways[9]. In recent years, the study of secondary metabolite production by *S. miltiorrhiza* hairy roots through the elicitation of physical and chemical treatments has been widely investigated with excellent results [10–14]. *Salvia miltiorrhiza* (also called Danshen in Chinese, belonging to Lamiaceae) is a famous medicinal crop worldwide and is extensively used for gynecology in traditional Chinese medicine (TCM). It has multiple functions, such as invigorating blood, nourishing blood, relieving menstruation and relieving pain. Two main components from *S. miltiorrhiza* have been reported. One is water-soluble phenolic acid compounds, such as rosmarinic acid and salvianolic acid B. The other is liposoluble tanshinone compounds, such as tanshinone IIA, tanshinone I, dihydrotanshinone and cryptotanshinone, that protect the myocardium and have anticancer effects [15,16].

5-Azacytidine (5-Az), a DNA demethylation agent, is a nucleotide analog that is used as a DNA methyltransferase inhibitor. 5-Az can selectively activate gene expression in eukaryotic cells and change the state of cell differentiation in specific cells (Christman, 2002; Jones, 1985). 5-Az was originally developed and tested as a nucleoside antimetabolite for acute myelogenous leukemia in humans. It also has antibacterial, antitumor, suppresses immunity, inhibits mitosis, protects against radiation, and inhibits virus replication [17,18]. The epigenetic mechanism of 5-Az is randomly incorporated into newly synthesized DNA strands where it irreversibly binds to DNA methyltransferases and then decreases DNA methyltransferase activity followed by genome hypomethylation at random sequences in a dose- and time-dependent manner [19,20]. 5-Az could induce various plant phenotype changes, including dwarfism, early flowering and inhibition of vegetative growth [21]. In addition, the stimulating effect of DNA methyltransferase inhibitors on the accumulation of secondary metabolites was also investigated[22].

In our study, the DNA demethylation agent 5-Az was used on the hairy root system of *S. miltiorrhiza* to systematically investigate the effects on secondary metabolite production via regulation of DNA methylation. The gene expression of key enzymes for phenolic acid and tanshinone compound biosynthesis in *S. miltiorrhiza* hairy roots was also addressed using sensitive qualitative and quantitative techniques. In terms of gene expression profiling of key enzymes under epigenetic characterization of the *CPS* promoter region, the underlying mechanism of tanshinone biosynthesis was further demonstrated. This is the first report to simultaneously demonstrate and compare phenolic acid and tanshinone compound biosynthesis in *S. miltiorrhiza* hairy roots under epigenetic modulation.

Results

Growth and morphology were affected by various 5-Az concentrations

Growth and morphological changes in *S. miltiorrhiza* hairy roots with different concentrations of 5-Az are shown in Fig. 1. The fresh weight and dry weight were measured after 28 days of treatment, and more significant growth retardation of hairy roots was shown for treatment with higher dosages of 5-Az. The fresh weight of hairy roots decreased by approximately 40% when the medium contained 12.5 and 25 μM 5-Az. The mass of hairy roots decreased by more than half when the medium contained greater than 50 μM 5-azacytidine.

Liposoluble active component production was increased by HPLC analysis

The active components in *S. miltiorrhiza* hairy roots were measured after treatment with different concentrations of 5-Az for 28 days, and the contents of liposoluble components increased significantly during the treatment period. However, 5-Az treatment did not show any effect or even decrease the production of water-soluble components in *S. miltiorrhiza* hairy roots. As illustrated in Fig. 2, the amounts of liposoluble components were not affected when the concentration of 5-Az was below 25 μM. In particular, the production of liposoluble components, including dihydrotanshinone I, tanshinone I, cryptotanshinone, tanshinone IIA and tanshinone IIB, was increased by 1.5 to 5 times compared with that of the control groups when the 5-Az concentration was greater than 50 μM (Fig. 2-A~E). In contrast to the liposoluble components, 5-Az decreased the production of water-soluble components in hairy roots at all concentrations. The contents of rosmarinic acid and salvianolic acid B declined by approximately 50% and 20% compared with the control groups, respectively (Fig. 2-F, G).

The regulation of gene expression was affected by 5-Az (via RT-PCR)

Gene expression in the biosynthesis pathway of secondary metabolites in *S. miltiorrhiza* was measured on different days with or without 5-Az treatment. 5-Az enhanced gene expression in the tanshinone-related compounds biosynthesis pathway but decreased most gene expression in the phenolic compounds biosynthesis pathway.

Two biosynthesis pathways are involved in the production of hydrophilic phenolic acids, namely, the phenylpropanoid pathway and tyrosine-derived pathway. In the phenylpropanoid pathway, the gene expression of PAL, C4H and 4CL1 declined by approximately 30%, 20% and 50%, respectively, when *S. miltiorrhiza* hairy roots were treated with 5-Az for 4 days. On day 7, the levels of PAL and C4H were upregulated to 38% and 24%, respectively, but 4CL1 gene expression still declined by approximately 60%. On day 14, there was little difference in the gene expression of PAL, C4H and 4CL1 compared with the control group. In addition, the gene expression level of HPPR in the tyrosine-derived pathway fluctuated from 0.7- to 1.3-fold during the 5-Az treatment period. The gene expression of TAT was

decreased by approximately half on day 1 but increased more than 2-fold from day 4 to day 14 compared with the control group (Fig. 3).

The mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in plastids are the two biosynthesis pathways that contribute to the accumulation of tanshinones in *S. miltiorrhiza*. As shown in Fig. 4, the level of HMGR in the MVA pathway was measured during the experiment and showed that 5-Az slightly increased its gene expression level by approximately 16% on day 1, but it gradually declined by approximately 30% over time. Moreover, most genes involved in the MEP pathway, including DXS1, DXS2, DXR, GGPPS, CPS and KSL, showed an upward trend when the hairy roots were cultured with 5-Az within 4 days. The gene expression of DXS2 was upregulated approximately 2.7-fold on day 1 and day 4 but dropped to 1.2-fold on day 7, and there was no difference on day 14 compared with the control group. DXR expression showed a similar trend to that of DXS2. The expression level increased by approximately 44% on day 1 and day 4 and then showed no difference after day 7. Positive regulation of the expression of GGPPS and CPS was also observed. The gene expression of GGPPS increased approximately 1.5-fold on day 1 and day 4, suddenly dropped to 0.7-fold on day 7 and was enhanced again to 1.4-fold on day 14. In contrast, the expression level of CPS was upregulated approximately 1.4-fold on day 1, declined to 1.1-fold on day 4, and then increased up to 1.5-fold on day 14. In contrast to the above-mentioned trend, the relative expression levels of DXS1 and KSL in the MEP pathway showed negative regulation. DXS1 was downregulated by approximately 30% on day 1 and day 4, and there was no difference after day 7. The relative expression of KSL also decreased by approximately 20% on average during the experiment.

DNA methylation was changed by 5-Az

The ratio of methylation levels in genomic DNA was measured on different days with or without 5-Az treatment. The results indicated that there was no significant difference in the methylation ratio on day 1 and day 4, which was only approximately 5% less than that of the control group. However, the DNA methylation level changed sharply after day 4. The ratio decreased by approximately 84% on day 7, while it increased by 27% compared with the control group on day 14 (Fig. 5). Therefore, 5-Az indeed exerted its inhibitory activity to influence the methylation ratio in genomic DNA.

5-Az alters DNA methylation at potential transcription factor binding sites

Based on the sequence information from Szymczyk et al. (2016), several putative transcription factors (TFs) might bind to specific motifs on the CPS promoter and enhance CPS gene expression. To prove that 5-Az might change the DNA methylation patterns in the promoter region, the methylation level of each cytosine on the CPS promotor was investigated. According to our experimental results, samples were collected on day 7 and processed by bisulfite conversion. Then, specific primers were designed to amplify

the promoter region. PCR products were analyzed by next-generation sequencing (NGS), and the results were investigated using PLANTPAN 3.0 [26] for potential TF binding sites. (Fig. 6).

Our results indicated that the methylation level of cytosine on the CPS promoter was altered during 5-Az treatment compared with the control group. One hundred forty-five cytosines were detected in our amplicons, among which fifty-one showed decreased methylation levels during 5-Az treatment. The regions of approximately 1468 bp to 1704 bp and 791 bp to 803 bp showed a greater alteration in demethylation levels compared with another region; moreover, cytosines at 803 bp, 1470 bp, 1655 bp, 1661 bp, and 1668 bp showed decreased methylation levels to 23.07, 25.01, 40.92, 30.78, and 26.14%, respectively (Table S1).

According to the analysis results, each cytosine might be recognized as having different TF binding, and thirty-six different TFs were identified at the demethylation sites, including alpha-amylase, AP2, ARR-B, AT-hook, B3, bet_v_1, bHLH, bZIP, C2H2, C3H, dehydhydrin, Dof, ERF, G2-like, GATA, GRF, HD-ZIP, homeodomain, LEA_5, MADS box, MIKC, M-type, MYB, MYB/SANT, NAC, NAM, NF-Y, RAV, SBP, TALE, TCP, tify, trihelix, WOX, WRKY, and ZF-HD. Among these TFs, NF-Y and MYB were frequently found at demethylation sites (Table 2).

Discussion

Plant secondary metabolites are specific compounds that are produced as part of the defense system and are mediators of interactions between plants and environments [27,28]. It has been reported that secondary metabolites have a critical responsive and regulatory relationship with DNA methylation in plants to adapt to environmental stresses. DNA methylation is recognized as an extensive epigenetic mechanism in plants. Pandey and Pandey-Rai [29] demonstrated that 3-hour UV-B treatment could upregulate the DBR2 gene and enhance the concentration of artemisinin up to 1.91-fold in *Artemisia annua*. Moreover, the global level of DNA methylation was reduced after UV-B treatment. This proved that the demethylation of DNA was responsive to UV-B. UV-B radiation also regulated flavonoid biosynthesis through epigenetic mechanisms in *A. annua*. Epigenetic characterization of the AaPAL1 promotor region in the flavonoid biosynthetic pathway revealed cytosine demethylation at specific sites in AaMYB1, AaMYC and AaWRKY TF binding sites under UV-B treatment. It caused overexpression of the AaPAL gene and significantly induced an increase in the total flavonoid content up to 2.4-fold compared to control plants [30]. Similarly, 5-azacytosine (5-AzaC) significantly increased the gene expression of stilbene synthase 10 (*VaSTS10*) and enhanced resveratrol production in *Vitis amurensis*. The DNA methylation level of the promotor and coding regions of the *VaSTS10* gene was decreased [31]. Based on previous research, changes in DNA methylation can significantly affect secondary metabolite production by regulating the expression of specific genes through their promotors.

In our experiment, 5-Az treatment significantly increased the content of tanshinones in *S. miltiorrhiza* hairy roots but reduced the content of water-soluble compounds, such as rosmarinic acid and salvianolic acid. Similar trends could also be observed in the gene expression levels. 5-Az treatment upregulated the

expression of specific genes responsible for tanshinone synthesis in the MEP pathway but downregulated the expression of genes related to hydrophilic phenolic acid biosynthesis. However, the opposite results were reported for water-soluble compounds when experiments were performed using different demethylation reagents, such as 5-AzaC, in *S. miltiorrhiza* hairy roots [22]. Their study indicated that the effect of demethylation by 5-AzaC could increase the contents of caffeic acid, rosmarinic acid and salvianolic acid in *S. miltiorrhiza* hairy roots. Demethylation reagents, such as 5-azaC (5-azacytosine), 5-az (5-azacytidine), 5-aza-2'-deoxcycytidine and 5,6-dihydroazacytidine, have been used to treat host cells to change DNA methylation levels, and their mechanisms are varied [32–34]. Moreover, the dosages of DNA methylation inhibitors might play an important role in regulating the biosynthesis of intermediate compounds in plant cells. We believe that different results might arise from the different epigenetic reagents and the concentrations that we used.

The alteration of methylation patterns in gDNA might affect the gene expression level and result in the accumulation of secondary metabolites in plants. Yang, et al. [22] indicated that the reduction of methylation levels on the rosmarinic acid synthase gene (RAS) promotor could induce gene expression involved in the biosynthesis pathway and then significantly enhance the production of water-soluble compounds in *S. miltiorrhiza* hairy roots. To prove the mechanism of 5-Az, we observed the gene expression and gDNA methylation levels on different days. The results indicated that 5-Az treatment could upregulate the tanshinone biosynthesis pathway and dramatically decrease the global methylation level.

Since 5-Az treatment was able to enhance the expression of CPS, a key enzyme in the tanshinone biosynthesis pathway, we analyzed the methylation profile in the promoter region by NGS. The methylation patterns on the CPS promoter were altered by 5-Az, and the level of methylation also varied during the treatment. This result indicated that there may be relationships between the gene expression of the CPS promoter and the demethylation effect of 5-Az. Based on the demonstration by Szymczyk et al. (2016), many TF binding sites can be observed in the CPS promoter region. We found that 5-Az promoted a demethylation effect on some TF binding sites, and approximately thirty-six different TFs were recognized by PLANTPAN3.0. Among them, MYB and NF-Y were frequently found in our results, as shown in Fig. 6 and Table 2.

TFs modulate gene expression by directly/indirectly binding to DNA sequences, and the numerous TFs in organisms represent vital characteristics in the regulation of cellular processes. One of the largest TF families in plants is MYB proteins. Four subfamilies can be classified based on the numbers of adjacent MYB repeats in the DNA binding domain, namely, 1R-MYB, 2R-MYB, 3R-MYB and 4R-MYB. The function of MYB proteins has been studied in various plant species, including *Arabidopsis*, cotton, and rice [35–37]. In previous studies with *S. miltiorrhiza*, MYB proteins have been recognized as a control factor in water-soluble and liposoluble compound production. For example, the overexpression of SmMYB9b can improve the production of tanshinones and related ingredients [38], and the overexpression of SmMYB2 enhances the production of water-soluble compounds, such as rosmarinic acid and salvianolic acid B [39].

The other common TF found in our results was nuclear factor Y (NF-Y), which is also known as a heme-associated protein/CCAAT box binding factor. NF-Y is composed of three subunits, NF-YA, NF-YB and NF-YC, and these subunits can regulate downstream genes individually or work as a protein complex. Several reports have found that NF-Y responds to embryogenesis, flowering time and abiotic stress in *A. thaliana* [40,41]. Based on our findings, especially of the demethylation effect on MYB and NF-Y binding motifs on the CPS promoter, further important investigations might be inspired. For instance, whether the demethylation effect of 5-Az could alter the affinity between TFs and their binding sites, whether the demethylation effect of 5-Az could modulate TF promoters, or whether the interaction between TFs and promoters under 5-Az treatment is worth exploring.

We believe that the function of 5-Az in *S. miltiorrhiza* hairy roots might alter DNA methylation patterns and cause changes in gene expression related to secondary metabolism; for example, tanshinone production was improved. In our study, the CPS promoter region was used as a target as we tried to find a linkage between the demethylation effect of 5-Az and tanshinone production. We found that some DNA sequence motifs tend to be demethylated during 5-Az treatment, but whether the demethylation effect of these DNA sequence motifs affects the affinity between DNA and TFs is still debatable. Regardless of whether the demethylation effect of the DNA sequence motifs on the CPS promoter was the cause or a consequence, our study provides evidence for the possibility of a novel metabolic engineering strategy to promote important secondary metabolite production in medicinal plants.

Materials And Methods

Chemicals and reagents

Gamborgs B-5 basal medium, sucrose, 5-azacytidine and tanshinone IIA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tanshinone I, dihydrotanshinone I, and salvianolic acid B were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (WUHAN, HUBEI, China). Rosmarinic acid was purchased from ChromaDex (Los Angeles, CA, USA). Cryptotanshinone was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). HPLC grade ethanol was purchased from Honeywell Chemicals (Seelze, Germany). HPLC grade acetonitrile was purchased from Fisher Scientific (Seoul, Korea).

Hairy roots culture

S. miltiorrhiza cultivar plants were purchased from Taiwan Seed Improvement and Propagation Station, Council of Agriculture, Executive Yuan, Taichung, Taiwan, and its hairy roots were induced from leaf explants by *A. rhizogenes* (LBA1334) infection. Hairy roots were maintained in phytohormone-free (0.5x) Gamborg's B5 liquid medium with 3% sucrose in the dark (pH 5.2 ± 0.1) at 25 ± 1°C and subcultured every 4 weeks. All of plant materials used in this study were comply with local and national regulations.

5-Azacytidine treatment

All experiments were carried out in a 125 mL flask inoculated with 0.2 g fresh *S. miltiorrhiza* hairy roots. Experimental treatments were processed by diluting 5-Az stock solution to different concentrations (6.25

μM, 12.5 μM, 25 μM, 50 μM and 75 μM) in (0.5x) Gamborgs B5 liquid medium with 3% sucrose. A group without any treatment was used as a control. The hairy roots were harvested after 1, 4, 7, and 14 days for qRT-PCR and global DNA methylation assays; 28 days for HPLC analysis; and 7 days for next-generation sequencing.

Extraction and high-performance liquid chromatography (HPLC) analysis

The hairy roots samples were harvested and weighed after 28 days of treatment and then processed using a FreeZone 6 Freeze Dry System (Labconco, USA) until reaching a constant dry weight. Samples were ground into powder and extracted with 80% ethanol (0.05 $mL \cdot mg^{-1}$) in an ultrasonic bath for 50 minutes. The extracts were filtered through a 0.45 μm PVDF membrane filter before HPLC analysis.

Chromatography analysis was performed with a Fortis C18 column (particle size 5 μm, 250×4.6 mm; Fortis, UK) connected to a photodiode array (PDA)-equipped Shimadzu 10AP HPLC System (Shimadzu, Japan). The flow rate was 1 $mL \cdot min^{-1}$, and the temperature was set at 30°C. Acetonitrile (A) and 0.5% aqueous acetic acid (v/v) (B) were used as the mobile phases. Gradient elution was performed with a linear gradient according to the following program: t = 0 min, 98% A; t = 80 min, 10%. The sample injection volume was 10 μL and different detection wavelengths were selected to compare its UV absorption and retention time for the identification of individual compounds. The detection wavelengths were 254 nm for tanshinone I and dihydrotanshinone I; 270 nm for salvianolic acid B, cryptotanshinone and tanshinone IIA; and 330 nm for rosmarinic acid.

RNA isolation and qRT-PCR

The expression of tanshinone biosynthesis genes, including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase I (DXSI), 1-deoxy-D-xylulose-5-phosphatesynthase II (DXSII), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), geranylgeranyl diphosphate synthase (GGPPS), copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), as well as phenolic acid biosynthesis genes, including phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate:CoA ligase 1 (4CL1), tyrosine aminotransferase (TAT), and 4-hydroxyphenylpyruvate reductase (HPPR), was investigated by quantitative real-time PCR in the hairy roots of *S. miltiorrhiza*. Ubiquitin was used as the reference gene. The specific primers were obtained from published articles [23,24]. Total RNA was extracted using a PureLink™ RNA Mini Kit (Invitrogen, USA), and cDNA was prepared with superscript IV VILO master mix (Invitrogen, USA) according to the manufacturer's instructions. Real-time quantitative PCR (qRT-PCR) was performed with 100 nM each of the forward and reverse primers, powerup SYBR green master mix (Applied Biosystems, USA), and 20 ng of cDNA template in a total volume of 10 μL. The amplification program was as follows: 50°C for 2 min, 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of genes was calculated by using ubiquitin as an endogenous control following the $2^{-\Delta\Delta Ct}$ (cycle threshold) method.

DNA isolation

Total DNA was extracted using a PureLink™ Plant total DNA purification kit (Invitrogen, USA) according to the manufacturer's instructions. Samples were extracted after 1, 4, 7, and 14 days of treatment in 75 μM 5-Az, and the samples without treatment were used as the control group. The DNA quality was measured based on the A260/280 and A260/230 parameters using NanoVue (GE Healthcare).

PCR

Based on the results of Szymczyk, et al. [25], specific primers were designed to construct the CPS promoter and are listed in Table 1. The PCR mixture contained 1 U of DNA polymerase (KAPA), 0.2 mM dNTPs, and 2 mM $MgCl_2$ in the reaction buffer. Ten nanograms of fresh DNA was used as template and the reaction was performed in a volume of 25 μL. The primer concentration was 0.4 μM. The PCR parameters were as follows: initial denaturation at 98°C for 1 min; 40 cycles of denaturation at 98°C for 1 min, primer annealing at 51°C for 1 min, and extension at 72°C for 40 sec, and final extension at 72°C for 3 min.

Measurement of DNA methylation levels

The level of DNA methylation was measured by a global DNA methylation assay kit (Invitrogen, USA) according to the manufacturer's instructions, and 50 ng of DNA was used in each reaction. In this assay, DNA was bound to strip wells with high DNA affinity, and the methyl group on the cytosine was detected by using antibodies. The level of methylation was quantified calorimetrically using the absorbance at 450 nm. A standard curve was generated from a range of 0.1–5% of the total methylation level, and the equation was generated using a second-order polynomial curve.

Bisulfite conversion

The gDNA samples from the control group and 5-Az-treated hairy roots were converted by a fast bisulfite conversion kit (ab117127) according to the manufacturer's recommendations. Bisulfite conversion was used as a tool to detect the methylation level in the DNA fragment due to specific changes in the unmethylated cytosine. Cytosine without methyl groups was converted into uracil, whereas methylated cytosine was unaffected.

Next-generation sequencing

To understand the methylation level of each cytosine in the CPS promoter, next-generation sequencing was performed. Bisulfite-converted DNA was used as the DNA template and amplified with the primers listed in Table 1. The conditions for PCR were described above, but the annealing temperature was changed to 50°C. PCR products were mixed in equal volume and ligated with adaptors. Afterwards, the mixture of PCR products was analyzed by Illumina MiSeq System sequencing, and the results were compared to the CPS promoter as a reference gene. The methylation level of each cytosine was calculated by CLC Genomics Workbench v10.

Declarations

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Author contributions

W.C. and B.Y. designed the study and B.Y. performed the experiments. M.L. and M.Li. helped with measurement and analysis of the data, and provided valuable discussions. B.Y. and W.C. wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Statement

This experimental research on plants and plant materials were comply with local and national regulations. This study also complied with institutional, national, and international guidelines and legislation.

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Tables

Table 1

Primers used in this study

Bisulfite Primer (5’-3’)	
BSP1-F	TTTATATATGGAAAAAAAGAAAATTGGAATTG
BSP1-R	TCATACAAAAATATAAAAAATACTACTCTCTCTC
BSP2-F	TTTAGTATAGYGTTTAAATTATGGTATGATATTATTG
BSP2-R	TCAAAAATAATTATATAAATACAACTAAACACC
BSP3-F	GTAATAAATGAGATGTATTATATAATATTAATGAGG
BSP3-R	AAAAAATCAACTATAAAATTCAAAATCTAAAC
BSP4-F	AATTATAGTTTTATTGAATGGATTTTTTGTTTAG
BSP4-R	ATCATATTTTTCTTTTTTCTACCTAAAATC
BSP5-F	TATTGGGTTTGTTTTATTTGGTTTTTG
BSP5-R	ACCATTTATTAATCAATTTCTTTCTAC
BSP6-F	GAGATYGTGGGATAAATAAAAAAGG
BSP6-R	TATATTAATCCATCATATTATACAATATTCCATTCAAC
PCR Primer (5’-3’)	
SM-F1	TCATAGAGAGAGTTGTCG
SM-R1	GGACAAAGAATCCATTCAG
SM-F2	GAGGTGTTCAGTTGTATC
SM-R2	CCTCCGTCTCATTAATCT
SM-F3	TGTCCCATTTGGTTTCTG
SM-R3	CGGGTTTAGGATTTGAGTT

Table 2

Demethylated cytosine sites in the CPS promoter and their corresponding homologous trans-factors from *A. thaliana* and other species.

Trans-factor name	Demethylated Cytosine Sites
Alpha-amylase	1685
AP2	537, 577, 1021, 1029, 1042, 1045, 1049
ARR-B	570, 572, 803, 1021, 1024
AT-Hook	537, 649, 651, 652, 729, 791
B3	537, 729, 1021, 1042, 1045, 1082, 1803, 1804
bet_v_1	570
bHLH	803, 1082, 1685, 1817
bZIP	136, 570, 708, 803, 1655, 1704, 1817
C2H2	626, 628, 942
C3H	803, 1672
Dehydrin	1162, 1165
Dof	577, 1661, 1668, 1672, 1685
ERF	577, 1029, 1049
G2-like	791
GATA	696, 1082, 1661
GRF	855
HD-ZIP	616, 708
Homeodomain	136, 570, 616, 708
LEA_5	803
MADS box	570, 1131, 1138, 1672
MIKC	1672
M-type	1672
MYB	235, 570, 572, 791, 1021, 1024, 1042, 1045, 1049
MYB/SANT	235, 570, 572, 791, 803, 1021, 1024, 1042, 1045, 1049, 1162, 1468, 1470, 1817
NAC	791, 803, 1668, 1672, 1685
NAM	791, 803
NF-Y	199, 241, 272, 572, 708, 729, 791, 855, 1024, 1049, 1138, 1162, 1165, 1440
RAV	537, 1021, 1042, 1045

SBP	1440
TALE	136, 570
TCP	272, 855, 942, 1440, 1655, 1661, 1668, 1672, 1690, 1817
tify	696, 1082, 1661
Trihelix	803, 1082, 1138, 1162
WOX	616
WRKY	136, 199, 570
ZF-HD	1470

Figures

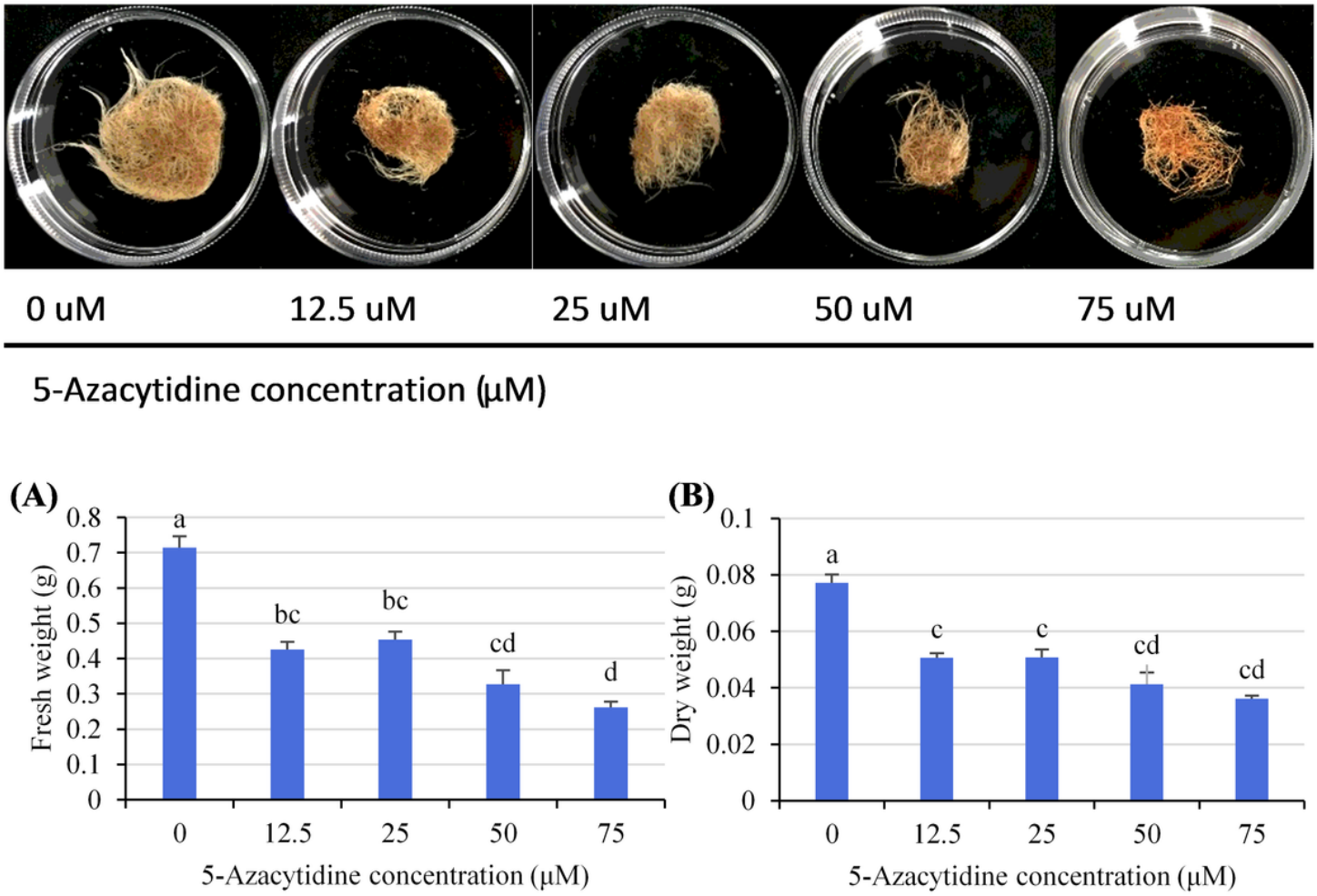

Figure 1

Influence of different concentrations of 5-azacytidine on Salvia miltiorrhiza hairy roots (A) fresh weight and (B) dry weight after 28 days of treatment. The values are the mean of at least three replicates ± S.E. Different letters indicate significant differences at the 5% level according to the LSD test.

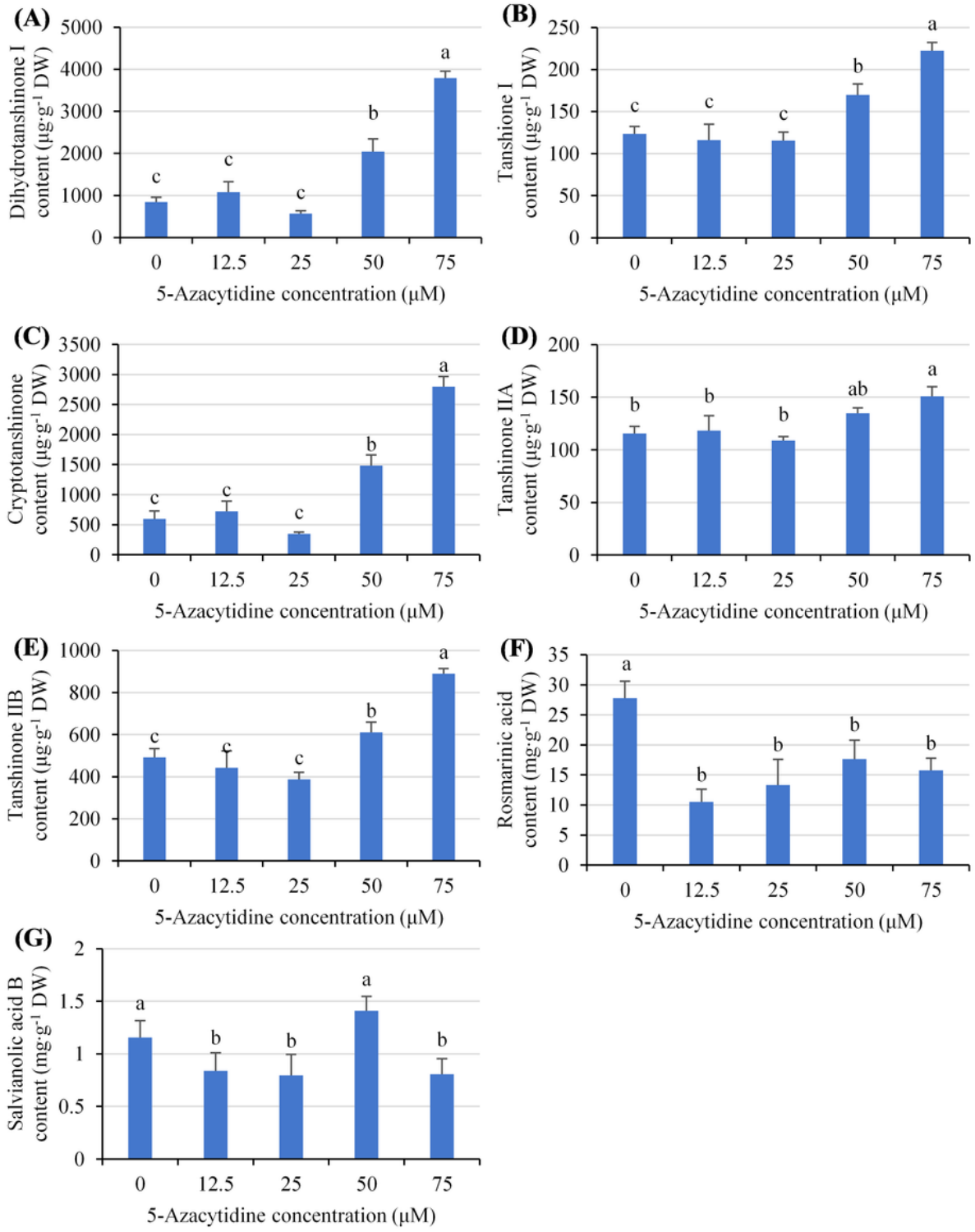

Figure 2

Contents of (A) dihydrotanshinone I, (B) tanshinone I, (C) cryptotanshinone, (D) tanshinone IIA, (E) tanshinone IIB, (F) rosmarinic acid, and (G) salvianolic acid B under different levels of 5-azacytidine in Salvia miltiorrhiza hairy root cultures after 28 days. The values are the mean of at least three replicates ± S.E. Different letters indicate significant differences at the 5% level according to the LSD test.

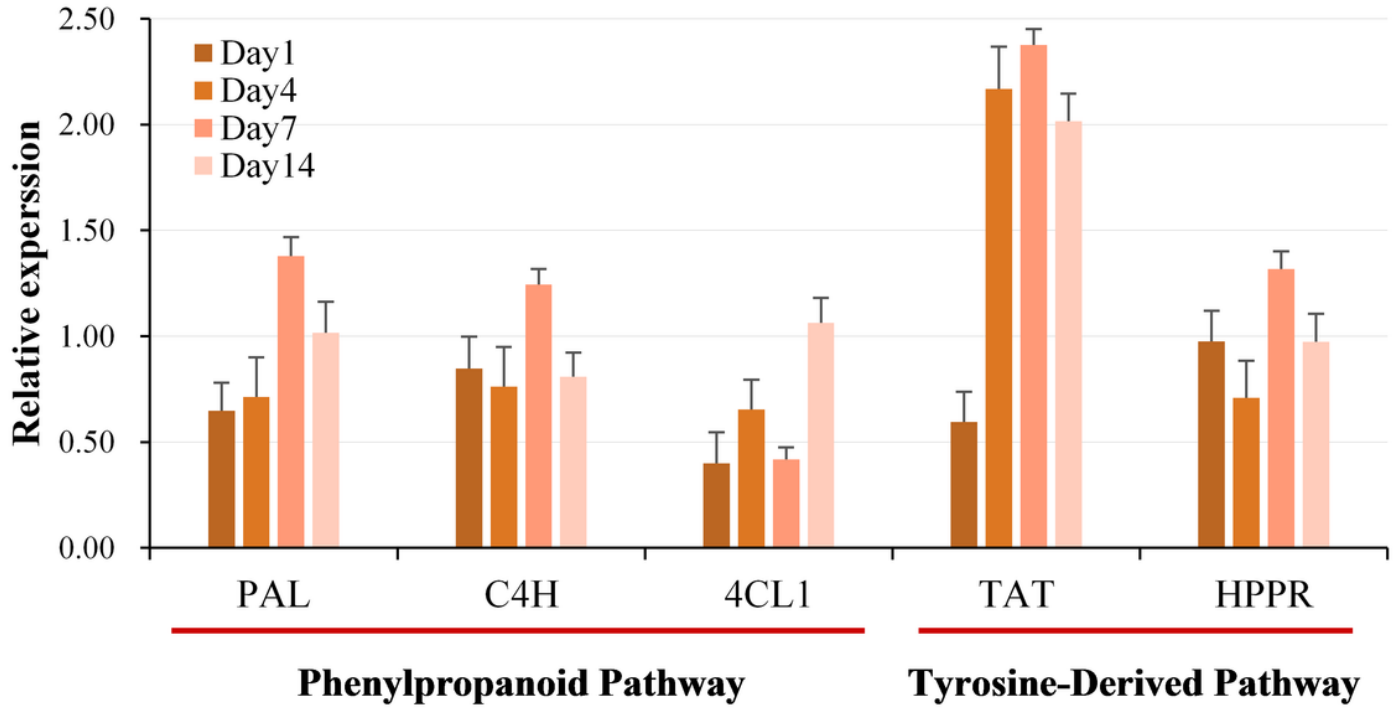

Figure 3

Gene expression of phenolic acid-related compounds in Salvia miltiorrhiza hairy roots at 1, 4, 7, and 14 days under 75 μM 5-azacytidine treatment. The values were calculated by using ubiquitin as the endogenous control, following the 2−ΔΔCt method. Bars represent the mean of relative expression ± S.E. of at least three replicates.

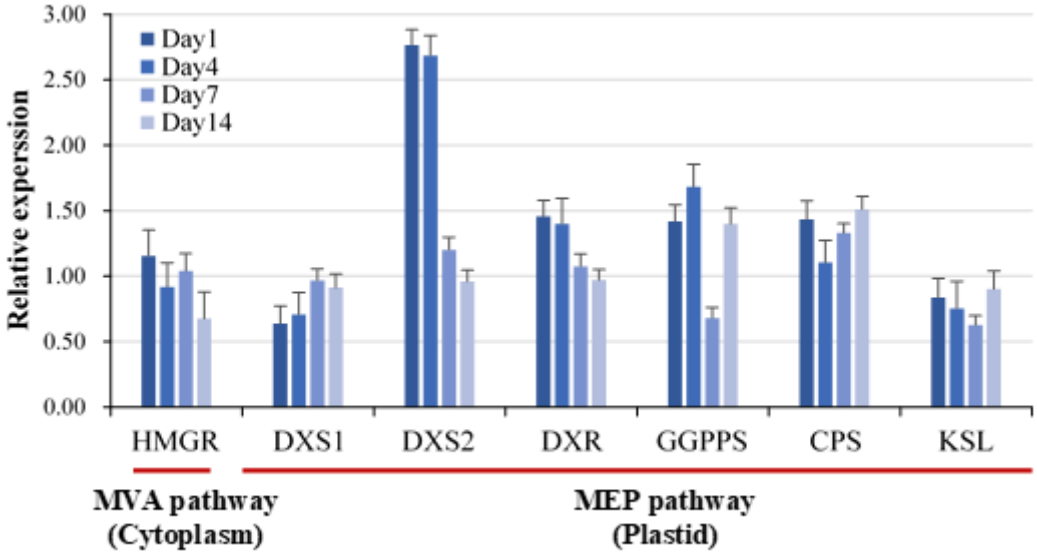

Figure 4

Gene expression of tanshinone-related compounds in Salvia miltiorrhiza hairy roots at 1, 4, 7, and 14 days under 75 μM 5-azacytidine treatment. The values were calculated by using ubiquitin as the endogenous control, following the 2−ΔΔCt method. Bars represent the mean of relative expression ± S.E. of at least three replicates.

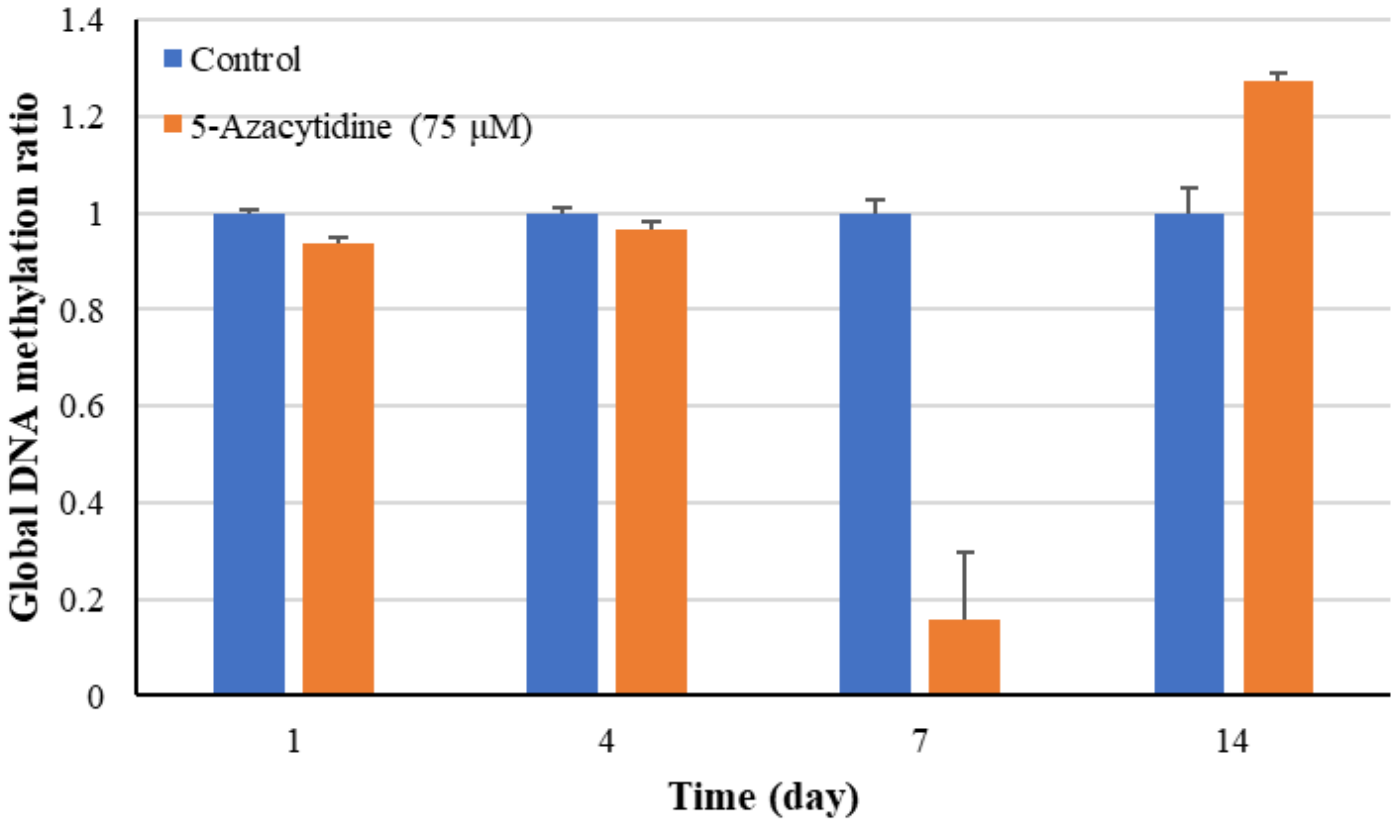

Figure 5

The ratio of global DNA methylation in Salvia miltiorrhiza hairy roots at 1, 4, 7, and 14 days with or without 75 μM 5-azacytidine treatment. The values are the mean of at least three replicates ± S.E. Different letters indicate significant differences at the 5% level according to the LSD test.

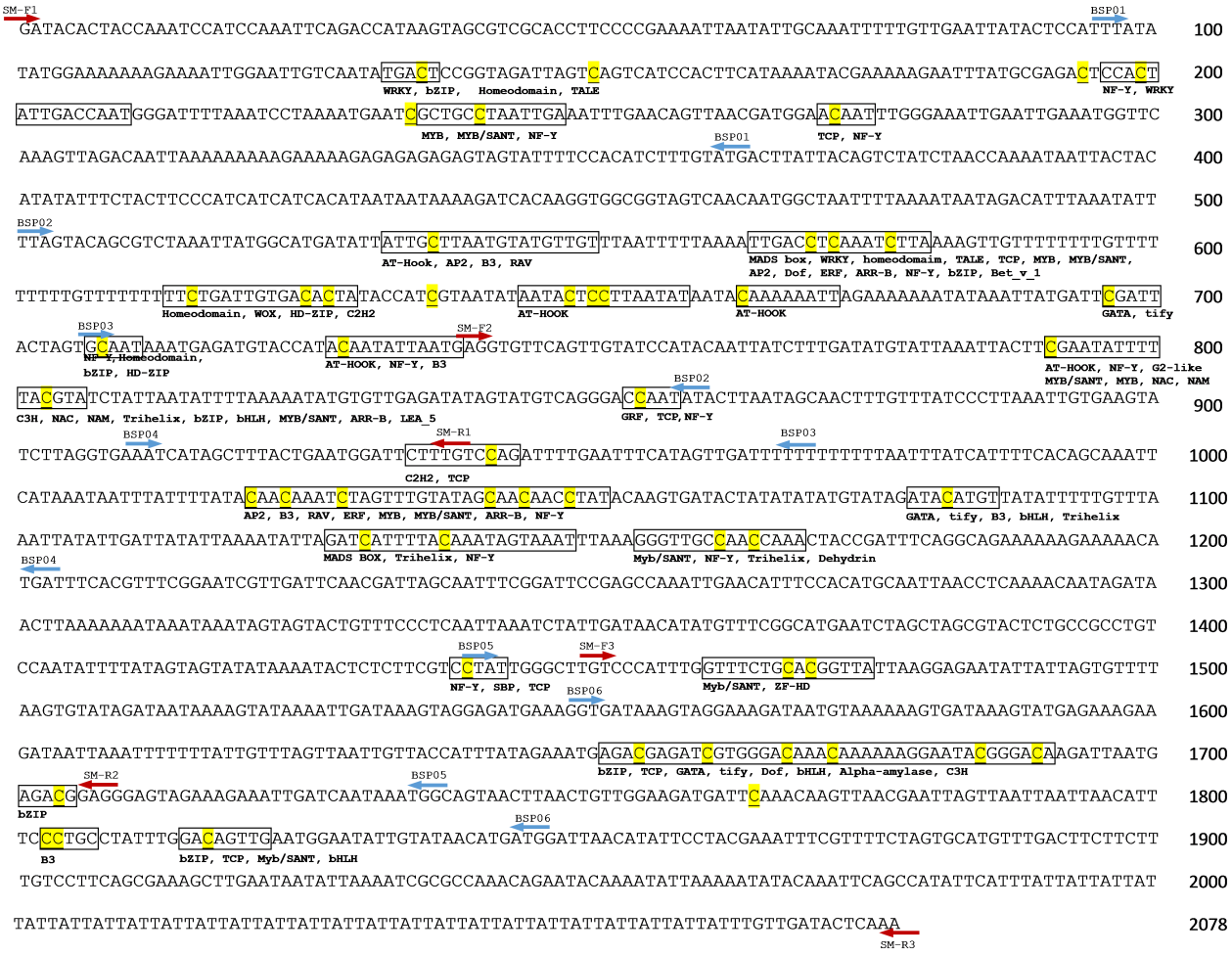

Figure 6

The S. miltiorrhiza CPS promoter region was amplified based on the reference from Szymczyk et al., 2016. Only the + strand is provided, and the specific primers are underlined. Compared with the control group, the demethylated cytosines are underlined in yellow, and the positions of transcription factors are boxed and indicated below.

Supplementary Files

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- graphicabstract.jpg