

# Function Analysis of Caffeoyl-CoA O-Methyltransferase for Biosynthesis of Lignin and Phenolic Acid in *Salvia miltiorrhiza*

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**Abstract** In this study, we cloned a full-length cDNA and the genomic DNA sequence of *SmCCoAOMT* (GenBank ID JQ007585) from *Salvia miltiorrhiza*. The 744-bp open-reading frame encodes a protein of 247 amino acids that shares 95 % similarity with one in *Vitis vinifera*. Real-time quantitative PCR analysis revealed that *SmCCoAOMT* is most highly expressed in the stems and can be induced by methyl jasmonate (MeJA) and XC-1 treatment. To evaluate its function *in vivo*, we generated RNA interference transgenic plants through *Agrobacterium tumefaciens*-mediated gene transfer. Compared with untransformed control plants, the transgenics had significantly less lignin and the expression of lignin-biosynthetic genes *SmCCR* and *SmCOMT* was depressed. In 90-day-old roots from plants of transgenic line M5, accumulations of rosmarinic acid and salvianolic acid B (Sal B) were greatly reduced by 0.89- and 0.69-fold, respectively. This low-Sal B phenotype was stable in the roots, with the level of accumulation being approximately 43.58 mg g<sup>-1</sup> dry weight, which was 52 % of the amount measured in the untransformed control. Our results suggest that *SmCCoAOMT* is involved in lignin biosynthesis and affects the accumulation of phenolic acids. This study also provides potential guidance for using lignin-related genes to genetically engineer *Salvia miltiorrhiza*.

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## Introduction

Lignin is a major component of secondary cell walls, providing mechanical support and also defending the plant against pathogen attack through increased lignification [1, 25]. Three types of lignin occur: guaiacyl (G), syringyl (S), and hydroxyphenyl (H) monolignol subunits, which are derived from *p*-coumaryl, coniferyl, and sinapyl alcohols [9]. The enzymes that catalyze lignin biosynthesis have been analyzed, and the genes involved in this biosynthetic pathway have been cloned and characterized in *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), and *Salvia miltiorrhiza* [2, 6, 27].

*Salvia miltiorrhiza* is one of the most famous traditional Chinese medicines because its constituent phenolic acids (especially salvianolic acid B, or Sal B) are effective in the treatment of cardiovascular diseases [13, 40]. The biosynthesis of lignin and phenolic acids follows the same phenylpropanoid pathway, converting phenylalanine to 4-coumaric acid. In *Salvia miltiorrhiza*, suppression of cinnamoyl CoA reductase (*CCR*) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (*COMT*) results in a reduction in lignin production while the concentrations of Sal B and another phenolic acid, rosmarinic acid (RA), are enhanced [34]. This process is consistent with that reported for other species [23, 31]. However, research with another lignin-related gene for caffeoyl-CoA O-methyltransferase (*CCoAOMT*) in *Arabidopsis thaliana* has shown that isorhamnetin is less abundant in the *ccomt 1* mutant, suggesting that the gene is involved in the methylation of flavonoids and phenolic acids [4]. Thus, all of those study results strongly imply that the role of *CCoAOMT* in the production of lignin and phenolic acids differs from that of other lignin biosynthesis-related genes.

*CCoAOMT* is specific for the methylation of caffeoyl CoA, a key metabolite in the biosynthesis of guaiacyl lignin. Various *CCoAOMTs* have been cloned from *Arabidopsis thaliana*, tobacco, *Zea mays*, and *Corchorus capsularis* [3, 16, 37]. However, little is known about such genes in *Salvia miltiorrhiza*.

In this study, we isolated and characterized the expression of *SmCCoAOMT* from *Salvia miltiorrhiza*. To gain insight into lignin and phenolic biosynthesis in this species, we applied RNA interference (RNAi) technology to suppress expression of that gene. Our goal was to provide a new perspective that could enable the engineering of improved phenolic acid production in this medicinal plant.

## Materials and Methods

### Plant Material and Culture Conditions

The mature seeds of *Salvia miltiorrhiza* Bunge were obtained from Shangluo (Shaanxi Province) and cultured on an MS basal medium. Plants of *Salvia miltiorrhiza* were grown in the greenhouse at  $25 \pm 2$  °C with a 16-h light/8-h dark photoperiod provided by cool-white fluorescent lamps ( $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ ).

## DNA and RNA Isolations

Genomic DNA and total RNA were extracted by the CTAB method [5] and Trizol reagent (BioFlux, China), respectively. cDNA was synthesized by reverse-transcription with oligo-dT primers (Takara, Japan).

## Cloning of *SmCCoAOMT* and Sequence Analysis

The full-length sequences of DNA and RNA for *SmCCoAOMT* were obtained from RNA-seq through PCR [12]. The 5' sequence end was cloned by DNA walking technology using a kit (no. 636405, Clontech, USA) (Table 1). PCR was performed as follows: 95 °C for 5 min; 34 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min with a final extension step of 72 °C for 10 min. Amplified fragments were then purified and sequenced (Takara, Japan). Similarity search of *SmCCoAOMT* were performed by BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple-alignment analyses were conducted using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/index.html>), and a neighbor-joining tree was built using PhyML [10]. Promoter was analyzed according to the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare.html>).

## Expression Analysis and Quantitative Real-Time PCR

Two-month-old plants were treated with either methyl jasmonate (MeJA, 5 μM) or the bacterium *Xanthomonas campestris* pv. *campestris* (XC-1). The growing conditions for XC-1 were previously described [18]. The expression of *SmCCoAOMT* was analyzed by real-time quantitative PCR, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) being amplified as a control (Table 1). PCR condition in the iQ5 thermocycler (Bio-Rad, USA) was as follows: 94 °C for 1 min, followed by 35 cycles at of 94 °C for 10 s,

**Table 1** Primer sequences

Primer	Sequence (5'–3')
SmCCoAOMT-full-F <sup>a</sup>	GAGAGATGGCGCAAACCTGCT
SmCCoAOMT-full-R <sup>a</sup>	GATCAGCTGATACGGCGGC
SmCCoAOMT-walking-F <sup>a</sup>	CTCTGGCTCTCTGGGATAGA CACTGG
SmCCoAOMT-walking-R <sup>a</sup>	TGCCCAACCTCCTGATGCCTGCC
SmCCoAOMT-F <sup>b</sup>	GGCATCAGGAGGTTGGGC
SmCCoAOMT-R <sup>b</sup>	CCGTCGTCGGGAAGAGCA
SmCCR-F <sup>b</sup>	TGCAAGGGGAAGGCACGTAT
SmCCR-R <sup>b</sup>	GGATGGGGCCAAGAAGCTGA
SmCOMT-F <sup>b</sup>	GCCACTAAGAATGTTGTCC
SmCOMT-R <sup>b</sup>	TCTGTCTTTCCTIACCA
GAPDH-F <sup>b</sup>	CCACCGTCCACTCCATCACT
GAPDH-R <sup>b</sup>	TGGGAACCTCGGAACGACATAC

<sup>a</sup>Used for cloning

<sup>b</sup>Used for RT-PCR

60 °C for 20 s, and 82 °C for 15 s of collection fluorescence. Expression was quantified by the comparative CT method [24].

### RNAi Construct and Plant Transformation

A 275-bp fragment corresponding to the 3' region of *SmCCoAOMT* cDNA was amplified by PCR, using upstream primer 5'-CCATCGATGGTACCCCTTGACATGGGGTTTCATACACCAG-3' (sites underlined for *Cla* I and *Kpn* I, respectively) plus downstream primer 5'-CGGGATCCTCGAGGCTGGGCTGATGAGGAATACTGCAAG-3' (sites underlined for *Bam*H I and *Xho* I, respectively). The *SmCCoAOMT* fragment was inserted upstream and downstream of the intron in the pKANNIBAL vector. The recombinant vector and pART27 [8] were ligated to generate the pART27-CCoAOMTi vector, and then introduced into *Agrobacterium tumefaciens* strain EHA105 [11].

Gene transfer was performed as described by Wang and Wang [33, 34]. To test for the putative transgenic plants, we amplified a 427-bp fragment from the intron, using primer PDK F (5'-GTGATGTGTAAGACGAAGAAG-3') and PDK R (5'-GATAGATCTTGCGCTTG-3'). The plasmid pART27-CCoAOMTi was used as positive control. The PCR-positive lines were used for further analysis.

### Lignin Analysis

Air-dried stems of transgenic and wild-type (WT) control plants were used for lignin analysis after being grown under the same conditions for 180 days. The stems were exhaustively extracted for 24 h with methanol and acetone separately in a Soxhlet apparatus. The main G and S lignin monomers (269 *m/z* for G and 299 *m/z* for S) were analyzed via gas chromatography–mass spectrometry (GC–MS; QP2010; Shimadzu, Japan) [38, 39]. Separation was achieved on an RTX-5MS column (30 m × 250 μm, 0.25 μm, Agilent J&W Scientific), with helium as the carrier gas at a constant flow rate of 1.19 mL/min. The temperature of injection, transfer interface, and ion source was set to 250, 250, and 200 °C, respectively. The GC temperature programming was set at 50 °C, followed by 35 °C/min oven temperature ramps to 220 °C, 0.5 °C/min to 230 °C, and 50 °C/min to 280 °C and a final 7-min maintenance at 280 °C. For relative quantification, peak areas from selected unique fragment ions for every identified compound were used. Levels of Klason lignin (acid-insoluble) and acid-soluble lignin were measured based on previous methods [38, 39].

### Extraction of Phenolic Acids and HPLC Analysis

Roots were collected from plants grown in the greenhouse for 90 or 180 days. Briefly, 20 mg of air-dried sample tissue was extracted with 75 % methanol (1 mL). Extractions of the phenolic compounds and analysis via high-performance liquid chromatography (HPLC; LC-2010A; Shimadzu) were performed as described by Zhang and Wang [34, 39].

### Statistical Analysis

Significant differences among results were evaluated by Tukey's pairwise comparison tests with SPSS 19.0 software. Results were considered to be statistically significant when  $p < 0.05$ .

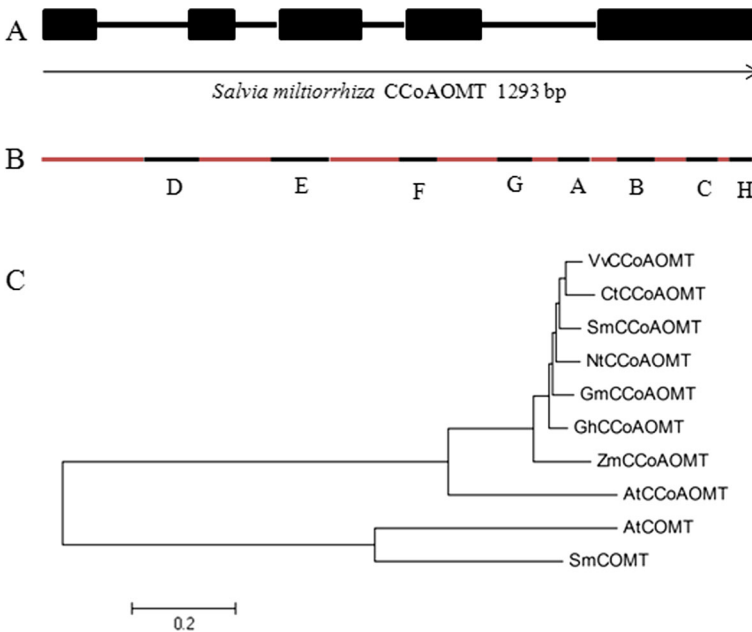
## Results

### Isolation and Characterization of *CCoAOMT*

Our PCR-based EST library and transcriptome analysis produced a 1037-bp sequence that we designated *Salvia miltiorrhiza CCoAOMT*. The full-length *SmCCoAOMT* was assigned with GenBank accession number JQ007585 (2783 bp). The open-reading frame was 744 bp long and the gene contained five exons and four introns in sizes of 549 bp (Fig. 1a).

The BLASTP analysis showed that the predicted protein shared high sequence identity with other CCoAOMTs, such as 95 % similarity with the protein from *Vitis vinifera*. The motifs of homologous CCoAOMT protein sequences were highly conserved and a comparison of active site residues presented five conserved domains and three catalytic domains (Fig. 1b). Molecular phylogenetic analysis demonstrated that *SmCCoAOMT* is very closely related to CCoAOMTs, including *Vitis vinifera* and *Carthamus tinctorius*. However, *SmCCoAOMT* and *SmCOMT* occupied separate categories, implying that they were involved in different methylation reactions (Fig. 1c).

Using genome walking, we obtained a 1320-bp 5' flanking sequence for *SmCCoAOMT*. Promoter analysis revealed several *cis*-acting regulator elements involved in light-responsiveness, including 4cI-CMA2b, an AE-box, ATCT-motif, G-box, L-box, and Sp1. The 5' flanking



**Fig. 1** **a** Structure of *SmCCoAOMT*. Exons are denoted by black boxes; introns, by lines. Lengths of exons are shown in base pairs. **b** Protein structure diagram: *DEFGH* conserved domains for  $Mg^{2+}$  and substrate binding; *ABC* conserved domains for methyl donor. **c** Phylogenetic tree of *SmCCoAOMT* and nine OMTs characterized from dicots and monocots. The tree was built using PhyML and neighbor-joining tree, with MEGA4. Accession numbers of sequences used for constructing tree are as follows: *Arabidopsis thaliana*, *AtCCoAOMT* (NP\_974104.1); *Arabidopsis thaliana*, *AtCOMT* (AT5G54160); *Carthamus tinctorius*, *CtCCoAOMT* (BAG71891.1); *Glycine max*, *GmCCoAOMT* (XP\_003518701); *Gossypium hirsutum*, *GhCCoAOMT* (ACQ59095); *Nicotiana tabacum*, *NtCCoAOMT* (Q42945.1); *Salvia miltiorrhiza*, *SmCOMT* (JF693491); *Vitis vinifera*, *VvCCoAOMT* (XP\_002282867); and *Zea mays*, *ZmCCoAOMT* (AAP37884.1)

regions also contained an AuxRR-box for auxin-responsiveness, plus TC-rich repeats that participate in defense and stress responses.

### Analysis of *CCoAOMT* Expression

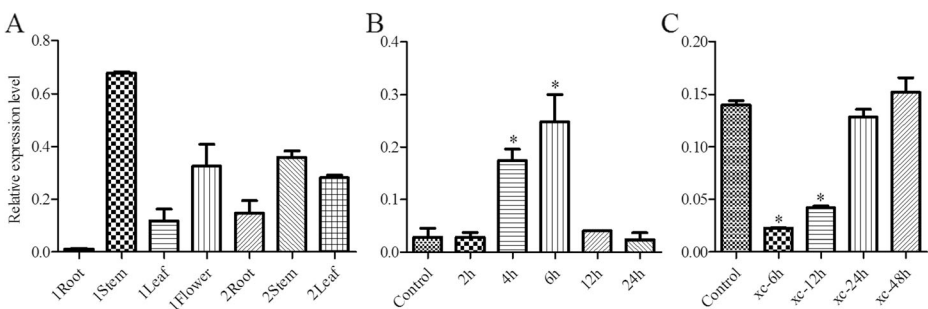
To gain further information about the spatiotemporal transcript abundance of *CCoAOMT* in *Salvia miltiorrhiza*, we performed tissue- and age-specific profiling via real-time quantitative PCR. Overall, *SmCCoAOMT* was expressed predominantly in the stems and at different growth stages. Expression was also high in the flowers (Fig. 2a). When compared with the untreated control, transcript levels were 11-fold higher in plants exposed to MeJA (Fig. 2b). Upon treatment with the XC-1 bacterium, transcription decreased in the first 6 h before rising at 24 and 48 h (Fig. 2c). This suggested that *SmCCoAOMT* participates in the plant defense mechanism.

### Development of RNAi Lines in *Salvia miltiorrhiza*

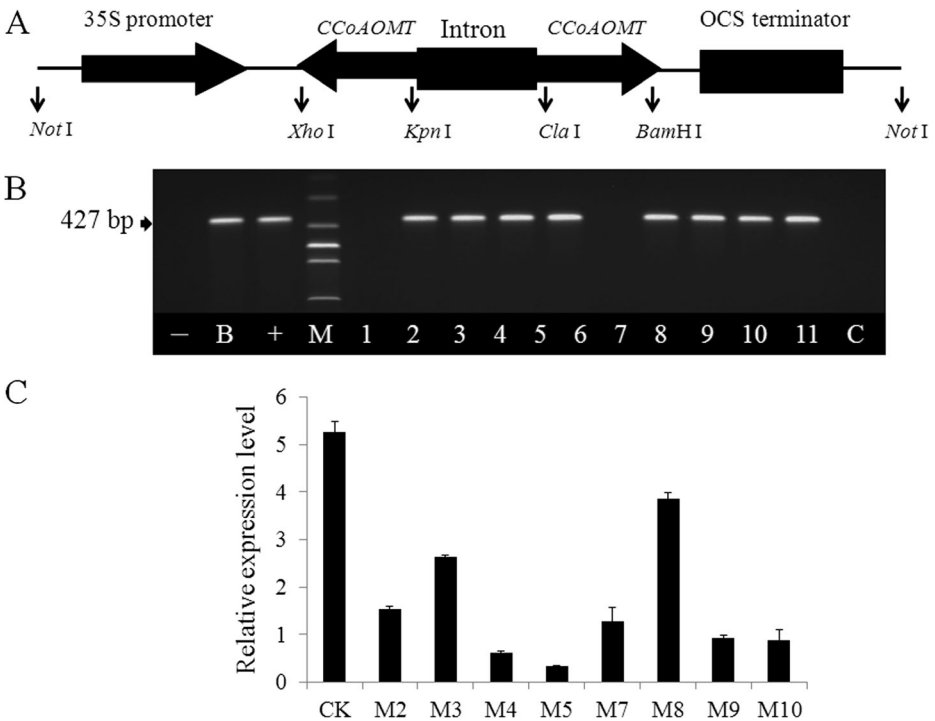
To understand the function of *SmCCoAOMT* in lignin biosynthesis, we constructed an RNAi vector that produced dsRNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3a). The *Agrobacterium*-transformed plants were cultured on a kanamycin medium, and 11 independent transgenic lines were selected. Leaves from 1-month-old plants were used for PCR analysis. An amplified 427-bp fragment of the intron confirmed that the transgenics from eight of those lines were positive (Fig. 3b, c). Expression of *SmCCoAOMT* was significantly decreased in all transgenic lines, especially lines M4 and M5, when compared with the control. However, no differences in phenotype were found among any of those lines (data not shown).

### Impact on Lignin Contents and Compositions

Our examination of the RNAi-transgenic lines and the control revealed changes in lignin levels and compositions (i.e., the relative proportions of S- and G-type monomers) (Table 2). Both the yield and relative frequency of the G monomers were reduced in the RNAi-transgenic stems while the S-lignin units were significantly higher. Consequently, the S/G thioacidolysis ratio was substantially higher in the transgenic tissues



**Fig. 2** Expression patterns of *SmCCoAOMT* in different organs (a) and under treatment with MeJA (b) or XC-1 (c). (1) Overwintering *Salvia miltiorrhiza* plant and (2) 2-month-old plants. Data represent averages of three experiments and error bars represent standard deviations. Asterisk represents values significantly different from untreated control at  $p < 0.05$



**Fig. 3** Vector construct and molecular analysis of transgenic plants. **a** PCR screening of *SmCCoAoMT*-suppressed *Salvia miltiorrhiza*. PDK intron was amplified to screen positive transgenic lines. Lane M, DL2000 DNA marker. Band sizes from top: 250, 500, 750, 1000, and 2000 bp; – control lines, B blank vector (without fragment of *SmCCoAoMT*), + positive control; lanes 1–11, different transgenic lines; C no-template control. **b** Sketch map of interfering box, including restriction sites and target gene. **c** Real-time quantitative PCR analysis of *SmCCoAoMT* in RNAi *Salvia miltiorrhiza*. All data are means of three replicates, with error bars showing standard deviations. Asterisk represents values significantly different from untransformed control at  $p < 0.05$

(0.89 for line M5 versus 0.69 for control). Finally, the contents of total lignin were decreased by 18.9 and 24.8 % in lines M4 and M5, respectively, when compared with the control plants (Table 2).

**Table 2** Lignin concentrations and compositions measured in mature stems of *Salvia miltiorrhiza* from M4, M5, and control lines<sup>a</sup>

Lines	Monomer yield (%) <sup>b</sup>			Lignin (mg g <sup>-1</sup> DW)		
	G	S	S/G	Acid-soluble lignin	Klason lignin	Total lignin
Control	59.1 ± 0.05	40.9 ± 0.04	0.69	0.77 ± 0.04	200.34 ± 4.81	201.01 ± 4.83
M4	53.6 ± 0.06*	46.4 ± 0.04*	0.86*	0.51 ± 0.03*	163.28 ± 5.42*	163.79 ± 5.44*
M5	52.9 ± 0.04*	47.1 ± 0.03*	0.89*	0.45 ± 0.03*	152.82 ± 5.93*	153.27 ± 5.94*

Data are means ± SD

\* $p < 0.05$

<sup>a</sup> Triplicate samples were analyzed for each line

<sup>b</sup> Measured by GC–MS

## Effect of *CCoAOMT* Inhibition on the Expression of *COMT* and *CCR*

The functional consequences of altering the expression of *CCoAOMT* was tested by measuring transcript levels of two genes related to the lignin-biosynthetic pathway in transgenic *Salvia miltiorrhiza*. Compared with activity in control plants, expression of *SmCOMT* and *SmCCR* was markedly reduced in the transgenics (Fig. 4). That pattern was consistent with the trend found for lignin contents.

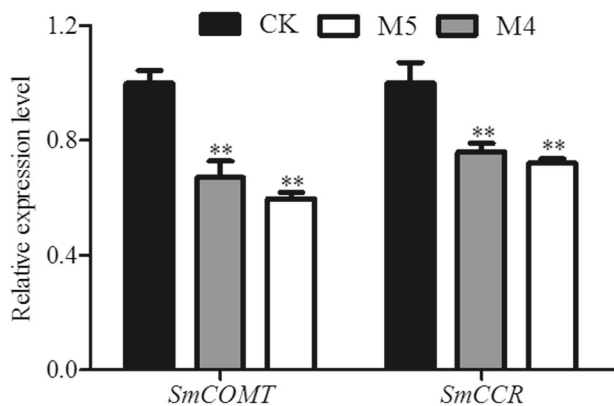
### Impact on Phenolic Acids

In addition to its established role in lignin formation, suppression of *CCoAOMT* can affect the metabolism of other participants in the phenylpropanoid pathway. To investigate whether such metabolism was modified in the RNAi-transgenic plants, we extracted their phenolic acids and separated them via HPLC. Levels of RA and Sal B, the two major hydrophilic and active pharmaceutical ingredients in *Salvia miltiorrhiza*, were decreased to varying degrees in 90-day-old plants from lines M4 and M5. Concentrations were lowest in the latter, i.e.,  $8.73 \pm 1.23 \text{ mg g}^{-1}$  dry weight (DW) for RA and  $38.90 \pm 0.72 \text{ mg g}^{-1}$  DW for Sal B. By comparison, those levels in control plants were  $9.88 \pm 0.78 \text{ mg g}^{-1}$  DW for RA and  $55.60 \pm 2.31 \text{ mg g}^{-1}$  DW for Sal B (Fig. 5a). To determine whether this low-RA/low-Sal B phenotype was stable during the stages of vegetative development, we examined the roots from genotypes after 180 days of growth and found that levels of RA and Sal B were further decreased by 34 and 47 %, respectively, in line M5 when compared with the control (Fig. 5b).

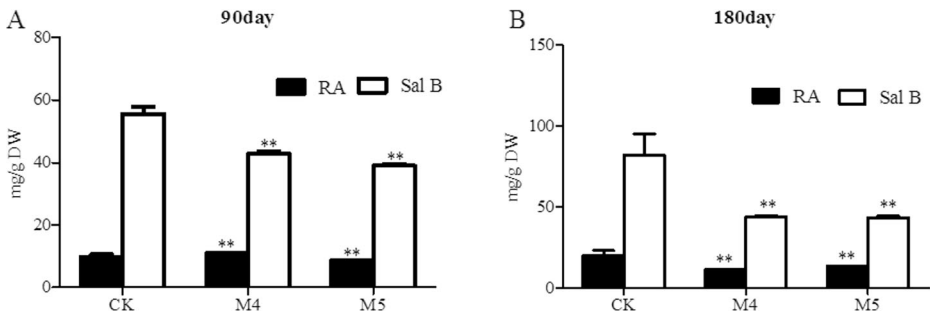
## Discussion

We have cloned a lignin-biosynthetic gene, *SmCCoAOMT*, from *Salvia miltiorrhiza* and evaluated its functioning in vivo. Cluster and conserved domain analyses indicated that roles vary between *CCoAOMT* and *COMT* genes, confirming that their roles differ for catalytic methylation during lignin biosynthesis in this species [14]. The expression pattern of *SmCCoAOMT* demonstrated that lignification was more active during that stage of development, with changes in transcripts following a pattern similar to that described for other *CCoAOMT*s [7, 16, 19]. We found it noteworthy that exposure to MeJA or XC-1 treatments

**Fig. 4** Relative expression levels of *SmCOMT* and *SmCCR* in control plants and transgenic lines M4 and M5. Data represent averages of three experiments and error bars represent standard deviations. \*\*values significantly different at  $p < 0.01$







**Fig. 5** Specific changes in levels of phenolic acids in transgenic *Salvia miltiorrhiza*. Concentrations of RA and Sal B in root samples from M4, M5, and control lines at 90 (a) and 180 days (b). All data are means of three replicates, with error bars indicating SD

enhanced the expression of *CCoAOMT*, thereby suggesting that lignin production is affected by signal transduction and plant biodefenses, consistent with results from promoter analysis. Similar findings have been reported for tobacco [16].

As observed previously with cinnamyl alcohol dehydrogenase (*CAD*), *CCR*, *COMT*, and phenylalanine ammonia lyase (*PAL*) [22, 30], lignin concentrations were reduced and compositions were altered in transgenic lines [29, 34]. However, a comparison of RNAi-*SmCCoAOMT* lines and control plants revealed no differences in phenotype. This is in contrast to the dwarfing phenotypes manifested by transgenic lines of *SmCCR* [34], indicating that *SmCCoAOMT* is not associated with lodging resistance [37]. In parallel, our investigation showed that downregulation of *SmCCoAOMT* led to decreased amounts of total lignin but an increase in the S/G ratio, which is consistent with previous study results [21, 32].

In contrast to other lignin-related genes in *Salvia miltiorrhiza* [4, 15], downregulation of *CCoAOMT* significantly decreased the accumulation of phenolic acids (RA and Sal B) in the transgenic plants, evidence that precursors not used in the lignin pathway were not reallocated. Those results from our experiments with RNAi-*SmCCoAOMT* are of interest because similar effects have been reported for other species [17]. One possible explanation is that *SmCCoAOMT* participates in the biosynthesis of phenolic acids by influencing the process of methylation. However, different genes in the lignin-biosynthetic pathway are not necessarily suppressed, implying that another potential metabolic branch may occur in the phenylpropanoid pathway [26, 28]. That aspect was not focused on in this current examination of *Salvia miltiorrhiza*, but the mechanism by which this phenomenon of lignin and phenolic acid deposition occurs will require further investigation.

Using genetic engineering to improve the production of effective components is becoming a new research goal with traditional Chinese medicines [35, 36, 39]. For example, some researchers have demonstrated that decreasing the flux through competitive pathways is an efficient strategy for increasing the levels of plant secondary metabolites [20]. Likewise, the synthesis of phenolic acids has been promoted by downregulating the expression of lignin synthesis-related genes in *Salvia miltiorrhiza* [34, 39]. Based on our studies, we propose that the functions of those genes should be comprehensively evaluated before taking this approach because blind application of similar strategies may lead to unexpected results. Nevertheless, our findings provide new insight into how *SmCCoAOMT* participates in the formation of health-promoting phenolic acids.

In summary, we cloned and characterized *SmCCoAOMT* from *Salvia miltiorrhiza* and analyzed its role in lignin biosynthesis. Our data showed that this gene influences the

accumulation of phenolic acids, especially Sal B. Therefore, we believe that *CCoAOMT* is a candidate gene for genetically engineering plants of this species to enhance their production of valuable phenolic acids.

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