Cloning of a caffeoyl-coenzyme A *O*-methyltransferase from *Camellia sinensis* and analysis of its catalytic activity[*]

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Abstract: Epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3"Me) present in leaves of *Camellia sinensis* has many beneficial biological activities for human health. However, EGCG3"Me occurs naturally in tea leaves in extremely limited quantities. Finding an enzyme from *C. sinensis* to catalyze the synthesis of EGCG3"Me is an alternative method to make up for the scarcity of EGCG3"Me in natural situations. In the present study, a complementary DNA (cDNA) encoding region and genomic DNA of the caffeoyl-coenzyme A *O*-methyltransferase (*CCoAOMT*) gene were isolated from *C. sinensis* (designated *CsCCoAOMT*). Nucleotide sequence analysis of *CsCCoAOMT* revealed an open reading frame of 738 bp that encodes a polypeptide with a predicted molecular weight of 28 kDa, which correlated well with the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The full-length DNA sequence (2678 bp) contained five exons and four introns. The deduced amino acid sequence of CsCCoAOMT shared 92% identity with CCoAOMTs from *Codonopsis lanceolata* and *Betula luminifera*. The catalytic activity of CsCCoAOMT was analyzed. Three monomethylated epigallocatechin-3-*O*-gallate (EGCG) compounds (EGCG4"Me, EGCG3"Me, and EGCG3'Me) were produced by CsCCoAOMT with K_m in the micromolar range. Real-time polymerase chain reaction (RT-PCR) experiments indicated that the *CsCCoAOMT* transcript was present at low levels during the early stages of leaf maturity (the first leaf and bud on a shoot) but the relative expression was augmented at advanced stages of leaf maturity (the third or fourth leaf on a shoot), which accorded well with changes in EGCG3"Me content in fresh leaves. Hence, we concluded that CsCCoAOMT catalyzes the syntheses of methylated EGCGs.

Key words: Tea (*Camellia sinensis*), *O*-methyltransferase, *CsCCoAOMT*, Prokaryotic expression, Catalytic activity, Methylated epigallocatechin-3-*O*-gallate (EGCG)

1 Introduction

Tea (*Camellia sinensis*) is consumed worldwide and its health-promoting effects have been widely explored (Lin and Lin-Shiau, 2006). Tea has many biological activities, such as anticancer (Jankun *et al.*, 1997), anti-obesity (Kuo *et al.*, 2005), and antibacterial properties (Bansal *et al.*, 2013; Keller *et al.*, 2013). Tea catechins are a group of polyphenolic compounds, which have been generally recognized as the major bioactive constituents responsible for the health properties of green tea. Epigallocatechin-3-*O*-gallate (EGCG), which accounts for almost 50% of the total catechins, shows the strongest biological activity in the vast majority of cases (Singh *et al.*, 2011). Recently, however, it was reported that some *O*-methylated EGCGs occurring naturally in tea leaves, such as epigallocatechin-3-*O*-(3-*O*-methyl)

gallate (EGCG3"Me) and epigallocatechin-3-*O*-(4-*O*-methyl) gallate (EGCG4"Me), showed much stronger anti-allergic (Maeda-Yamamoto *et al.*, 2004; 2007) and anti-hypertensive effects (Kurita *et al.*, 2010) than EGCG. Moreover, *O*-methylated EGCG (for example, EGCG3"Me) was absorbed more easily than EGCG (Sano *et al.*, 2000). Therefore, these *O*-methylated EGCGs have attracted much attention in recent years, especially EGCG3"Me. Unfortunately, EGCG3"Me occurs in extremely limited quantities, and very few tea genotypes contain >1% EGCG3"Me. Consequently, increasing the supply of *O*-methylated EGCGs by extraction from tea tissues is difficult; therefore, it is necessary to find alternative methods for efficient synthesis of EGCG3"Me.

Methylation reactions occur ubiquitously in all organisms. It is well known that *O*-methyltransferases (OMTs) are extremely important enzymes in plants that have significant regulatory effects on growth and development. Plant OMTs are generally categorized into two families according to their distinct substrate preferences and amino acid sequence homology, namely PI-OMT I and PI-OMT II. The lengths of PI-OMT I proteins vary from 231 to 248 amino acids and their activities are dependent on the presence of divalent cations. The lengths of PI-OMT II enzymes are 344–383 amino acids and, unlike the PI-OMT I family, the activities of PI-OMT II enzymes do not require Mg^{2+} ions. Caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT) belongs to the PI-OMT I family (Joshi and Chiang, 1998). It is well known that CCoAOMT is a key enzyme in the biosynthesis of lignin and catalyzes the methylation of caffeoyl-CoA to feruloyl-CoA in guaiacyl lignin biosynthesis. As an *S*-adenosyl-L-methionine (SAM)-dependent OMT, CCoAOMT transfers the methyl groups of SAM to the aromatic ring of monolignols (Zhao *et al.*, 2004). Its amino acid sequence contains eight conserved motifs. Motifs A, B, and C are common in OMTs, which are considered to be putative SAM-binding motifs, whereas motifs D, E, F, G, and H are the signature sequences of CCoAOMT (Hoffmann *et al.*, 2001).

It has been reported that some of the CCoAOMT may utilize both flavonoids and caffeoyl-CoA (Kim *et al.*, 2010a; 2010b). EGCG is a flavanol, which is a class of flavonoids. Consequently, CCoAOMT in plants probably shows catalytic activity in the methylation of EGCG. Kirita *et al.* (2010) cloned a novel

OMT from *C. sinensis* and several *O*-methylated EGCGs were synthesized by the recombinant enzyme. The enzyme was proposed to be a CCoAOMT. In the present study, a *CCoAOMT* gene in *C. sinensis* (*CsCCoAOMT*) was cloned and expressed in *Escherichia coli*. The gene structure, catalytic activity, and kinetic parameters of CsCCoAOMT were analyzed. Furthermore, we compared the relative expression level of *CsCCoAOMT* with EGCG3"Me content in fresh leaves of four tea cultivars.

2 Materials and methods

2.1 Plant materials, plasmids, and standards

Plant materials: four cultivars T1, T2, T3, and T4 grew in the tea plantation of the Tea Research Institute of the Guangdong Academy of Agricultural Sciences, China. Fresh leaf samples comprising four leaves and a bud were plucked from the four cultivars in July 2012. All leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C.

E. coli strains and plasmids: *E. coli* BL21 (DE3) and *E. coli* DH5α (TransGen Biotech, China), pET-51b(+) vector (Novagen, USA), cloning vector pMD18-T (TaKaRa, China). All strains were grown in Luria-Bertani (LB) medium.

Standards of EGCG3"Me, EGCG4"Me, and EGCG3'Me were kindly supplied by Taiyo Kagaku Co., Ltd. (Japan).

2.2 Cloning of *CsCCoAOMT*

Total RNA was extracted from leaf samples using the TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen, USA), and the integrity and quality of the RNA were checked according to Mamati *et al.* (2006). First-strand complementary DNA (cDNA) was synthesized using 1.0 μg total RNA with SuperScript Reverse Transcriptase (Invitrogen, USA). Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Khan *et al.*, 2004). Two primers (CSF 5'-ATGGCAACAAACGGAGAAGGA-3' and CSR 5'-GCAGACACGCCGGCACAG-3'), used for polymerase chain reaction (PCR) amplification of the *CsCCoAOMT* cDNAs and genomic DNA, were designed and synthesized according to the published cDNA sequence (GenBank Accession No.

DD361102). The PCR reactions were performed with Phusion DNA polymerase (Finnzymes, Finland) in three steps: (1) pre-denaturation at 95 °C for 5 min; (2) 35 amplification cycles at 95 °C denaturation for 30 s, annealing at 61 °C for 45 s and extension at 72 °C for 1 min (for cDNA amplification) or 3.5 min (for genomic DNA amplification); and, (3) post-extension at 72 °C for 10 min. The PCR products were analyzed in 1.5% (0.015 g/ml) agarose gel and the bands containing the target fragments were purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotech, China). 'A' tails were added to the purified fragments.

The purified DNA fragments with 'A' tails were inserted into the cloning plasmid pMD18-T vector (TaKaRa, China) and mixed with T4 DNA ligase at 16 °C for 5 h. All the connected products were inserted into the competence *E. coli* DH5α. The cultures were placed on ice for 30 min, given a heat shock at 42 °C for 1 min, and then transferred to an ice bath for 5 min, followed by culturing on solid LB medium with 100 μg/ml ampicillin at 37 °C overnight.

After clones were detected with M13 primers, positive clones were selected and multiplied in liquid LB medium supplemented with ampicillin for 12 h. Plasmid DNAs were extracted with the EasyPure Plasmid MiniPrep Kit (TransGen Biotech, China).

2.3 Sequence analysis

DNA sequencing was performed by Invitrogen, Shanghai, China. Sequence analyses were performed online (http://www.ncbi.nlm.nih.gov/). The BLAST and BLASTP programs were used for the sequence homology search. By comparison of the cDNA sequences with the genomic DNA sequences, the introns and exons of *CsCCoAOMT* were clear and definite. Amino acid sequences of CsCCoAOMT, CCoAOMT from 11 other plant species and CsOMT (GenBank Accession No. DD361102) were aligned using DNAMAN software.

2.4 Prokaryotic expression and purification of CsCCoAOMT

CsCCoAOMT was originally cloned in the pMD18-T plasmid. Based on the cDNA coding sequence of *CsCCoAOMT*, a pair of primers that contained two restriction sites, *Kpn*I and *Sal*I, were designed. *Kpn*I and *Sal*I sites were introduced by PCR at

the 5' and 3' ends of the *CsCCoAOMT* sequence using the following primers: 5'-GG<u>GGTACC</u>CATGGCA ACAAACGGAGAAGGA-3' (CS51F, *Kpn*I site underlined) and 5'-ACGC<u>GTCGAC</u>GCAGACACGCC GGCACAG-3' (CS51R, *Sal*I site underlined). The digested products were inserted into the expression plasmid pET-51b(+) mixed with T4 DNA ligase at 16 °C for 12 h. The pET-51b(+) plasmid was also digested with *Kpn*I and *Sal*I. The connected products were transformed in *E. coli* BL21 (DE3) and cultured in LB medium at 37 °C overnight, then subjected to restriction enzyme digestion and sequencing analysis.

The cells containing pET-51b-CCoAOMT were selected and cultured in LB (Amp[+]) medium for about 5 h, then the culture liquid was added to 500 ml LB (Amp[+]) liquid medium in the proportion of 1:100 (v/v) for further culture. When the culture liquid reached an absorbance of 0.6 at 600 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mmol/L to induce expression of the fusion protein. After 16 h of induction at 20 °C, the bacterial cells were collected, resuspended in histidine (His)-tag binding buffer (20 mmol/L NaH$_2$PO$_4$, 500 mmol/L NaCl, pH 7.4), and lysed by sonication. Crude enzymes (5 ml) were added into a His-tag affinity column (Amersham Biosciences, USA), which had been balanced with 20 mmol/L phosphate-buffered saline (pH 7.4) for 10 min. The His-tag affinity column was stored at 4 °C for 2 h, then washed with an imidazole buffer concentration gradient (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, and 500 mmol/L). The eluent was collected in different tubes and the absorbance rate determined at 280 nm. Those tubes showing absorbance at 280 nm were selected, and the selected samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (0.12 g/ml) polyacrylamide gel. Thus, the optimal concentration for eluting the fusion protein was ascertained.

2.5 Enzyme assays, product identification, and kinetic analysis

Enzymatic reactions were performed using the purified recombinant protein. To evaluate the enzyme activity of CsCCoAOMT, 0.2 mg purified protein was added to a 15-ml reaction system including 0.05 mmol/L EGCG, 0.16 mmol/L SAM, 0.2 mmol/L MgCl$_2$, and 100 mmol/L Tris-HCl (pH 6.8). The

reaction mixture was incubated at 35 °C for 30 min, before the reaction was terminated by addition of 1 mol/L HCl. After the mixture was extracted with 30 ml ethyl acetate, the organic phase was evaporated to dryness, and the residue was resuspended in 1 ml ultra-pure water. Kinetic analyses were performed within the linear range of reaction velocities by adjusting the concentrations of EGCG (50 to 500 μmol/L) and SAM (80 to 400 μmol/L). All the methylated EGCGs are used as products for determining the kinetic parameters.

The resuspended products were analyzed using an Agilent Infinity 1290 high-performance liquid chromatography (HPLC) equipped with an Agilent 6530 QTOF mass spectrometer with a dual electrospray ionization source in the positive ionization mode. A Waters SunFire C18 column (150 mm×2.1 mm internal diameter, 5 μm particle size) was selected as the stationary phase, and the column temperature was set at 40 °C. The binary mobile phase was 0.5% formic acid solution including 2 mmol/L ammonium formate (A) and acetonitrile (B). The gradient elution began with 12% solvent B, increased linearly to 25% solvent B over 5 min, and continued to increase to 75% solvent B over 15 min before decreasing to 12% solvent B over 5 min. The flow rate was 0.3 ml/min and the injection volume was 5 μl. The mass accuracy was controlled through online calibration using reference ions (m/z 121.0509, 922.0098). The capillary voltage, temperature, nebuliser pressure, and flow rate of the drying gas were set to 3.5 kV, 300 °C, 35 psi (1 psi= 6.895 kPa), and 8 L/min, respectively. Mass spectra were recorded for the m/z range of 100–1000. For accurate identification, the methylated products were subjected to the accurate mass, mass patterns, and retention time comparison with authentic standards.

2.6 Analysis of *CsCCoAOMT* expression and EGCG3″Me content

The relative abundance of the *CsCCoAOMT* transcript was measured by real-time PCR (RT-PCR) at four stages of leaf maturity (the first leaf and bud, the second leaf, the third leaf, and the fourth leaf from the shoot apex) of four tea cultivars (T1, T2, T3, and T4) that differed greatly in EGCG3″Me content in fresh leaves. The primers P3-R (reverse, 5'-ACACT GGTTACTCTCTTCTCG-3') and P3-F (forward, 5'-GATGTAGTTGTCCTTGTCAGC-3') were de-

signed on the basis of the cDNA sequences. In addition, the 18S rRNA gene, amplified with the primers 18S rRNA-R (reverse, 5'-GCTGGAATTACCGCGG CT-3') and 18S rRNA-F (forward, 5'-CGGCTACCA CATCCAAGGAA-3'), was used as an internal reference gene.

RT-PCR was performed with the 7500 Real-Time PCR System (Applied Biosystems, Inc., CA, USA) with the following settings: 95 °C for 60 s; 40 cycles of 95 °C for 30 s, 60 °C for 34 s, and 72 °C for 30 s. PCR reactions were carried out in triplicate on a 96-well plate with 50 μl per well using SYBR Premix Ex Taq (TaKaRa, China). The relative quantification values for each gene were calculated with the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Ma *et al.*, 2012). Data represent the average of three experiments.

The EGCG3″Me contents in leaves at four stages of maturity for each of the four tea cultivars were detected by HPLC according to the methods of Lv *et al.* (2008). A Waters 2489-2690 HPLC was used and samples were separated using a Waters SunFire C18 column (150 mm×2.1 mm, 5 μm).

3 Results

3.1 Isolation of *CsCCoAOMT* gene

The cDNA (GenBank Accession No. JQ790527) and genomic DNA (GenBank Accession No. JQ790528) of *CsCCoAOMT* gene were cloned. Sequence analysis of *CsCCoAOMT* showed that the open reading frame (ORF) contained 738 bp (Fig. 1a), which encoded a polypeptide of 245 amino acid residues (Fig. 2). The genomic DNA of *CsCCoAOMT* was 2678 bp in length and comprised five exons and four introns (Figs. 1b and 1c), which was in concordance with the maize and *Eragrostis curvula CCoAOMT1* gene intron-exon arrangement. In contrast, three introns were identified in rice *CCoAOMT* and maize *CCoAOMT2* (Guillet-Claude *et al.*, 2004).

3.2 Sequence analysis of CsCCoAOMT

Amino acid sequences of CsCCoAOMT, CCoAOMT proteins from 11 plant species, and CsOMT from *C. sinensis* were aligned using DNAMAN software. High homology between CsCCoAOMT and the other CCoAOMT proteins was apparent; for example, the predicted CsCCoAOMT

protein sequence showed 92% identity with CCoAOMT proteins from *Codonopsis lanceolata* and *Betula luminifera*, and 87% similarity with CCoA-OMT proteins from *Gossypium hirsutum* and *Pinus pinaster*. Homology between CsCCoAOMT and CsOMT from *C. sinensis* was 99%. There were two amino acid differences between the CsCCoAOMT and CsOMT reported by Kirita *et al.* (2010). They were cloned from different tea genotypes. All regions were highly conserved except the 5' end (Fig. 2). CsCCoAOMT contained all conserved motifs, i.e., the motifs A, B, C, D, E, F, G, and H. Motifs A, B, and C are common in plant OMTs and are thought to be specific SAM-binding sites. The specific spatial arrangement might be essential for the formation of the SAM-binding pocket (Joshi and Chiang, 1998; Zhao *et al.*, 2004). Additional CCoAOMT signature motifs were indicated by D, E, F, G, and H.

3.3 Expression and purification of CsCCoAOMT

CsCCoAOMT was expressed successfully in *E. coli* strain BL21 (DE3) after induction of expression by IPTG for 16 h. SDS-PAGE analysis showed that the relative molecular mass of the CsCCoAOMT fusion protein obtained was approximately 28 kDa, as expected, and was expressed both in the supernatant and sediment (Fig. 3a). After purification with a His-tag affinity column and washing with an imidazole buffer concentration gradient, 200 mmol/L imidazole buffer was the optimal concentration for eluting the fusion protein. Hence, the target protein was collected

in 200 mmol/L imidazole buffer, and then analyzed by SDS-PAGE (Fig. 3b). The purified enzyme was used to verify its catalytic activity.

3.4 Catalytic activity and kinetic parameters of CsCCoAOMT

The products of the enzymatic reaction (see Section 2.5) were analyzed by HPLC coupled to quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). No methylated products were detected in the control reactions (heated CsCCoAOMT, Fig. 4b), whereas with the addition of CsCCoAOMT, three monomethylated EGCG products were detected (Fig. 4a). The retention times (t_R) of these products (A, B, C) were 8.40, 8.61, and 9.10 min (Fig. 4a). Identical to the monomethylated EGCG standards, the m/z of all three products was 473.1078, which was 14.1058 higher than the m/z of EGCG (459.0020). From comparison of the ion chromatograms, it was clear that each t_R of products A, B and C was consistent with the t_R of EGCG4"Me, EGCG3"Me, and EGCG3'Me, respectively (Fig. 4c). The MS patterns of the three products were also similar to those of the standards (Figs. 4d and 4e). The deviation between the actual and theoretical m/z values of all detected compounds was less than 5×10^{-6}. These results indicated that the three products were identifiable as EGCG4"Me (compound A), EGCG3"Me (compound B), and EGCG3'Me (compound C). Therefore, it was concluded that CsCCoAOMT catalyzes the synthesis of methylated EGCG.
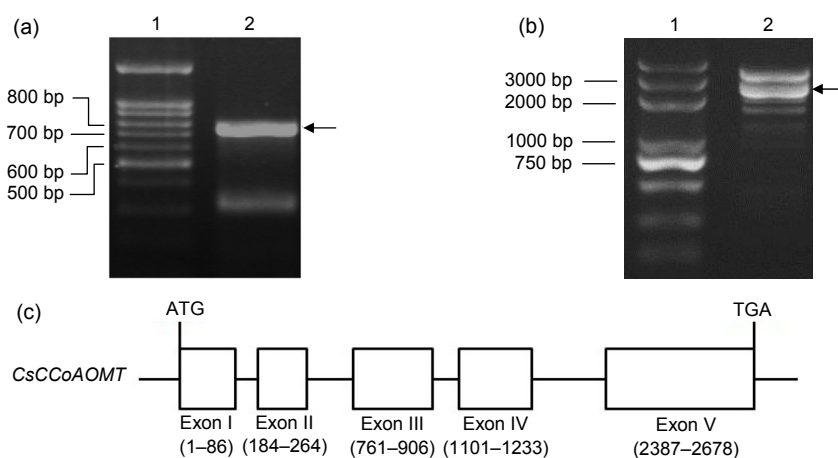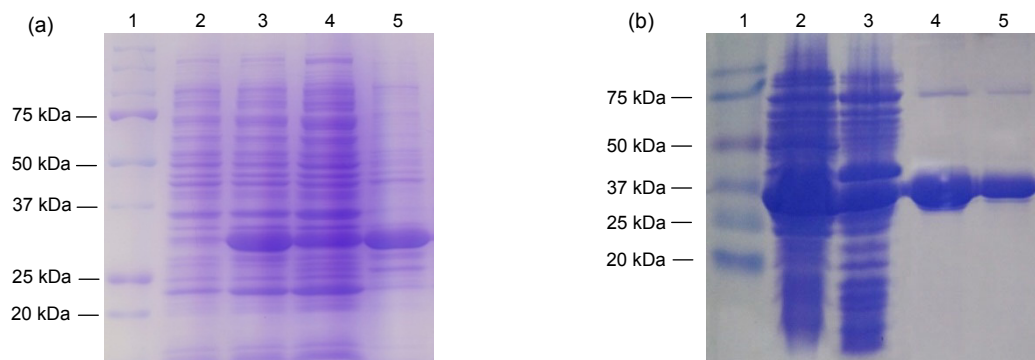

Fig. 1 Analysis of *CsCCoAOMT* gene

The target products are indicated with black arrows. (a) Amplification of a cDNA ORF of *CsCCoAOMT*. 1, marker; 2, PCR product. (b) Amplification of genomic DNA of *CsCCoAOMT*. 1, marker; 2, PCR product. (c) Schematic representation of the structure of *CsCCoAOMT*. Five exons with their initial and final nucleotides are shown
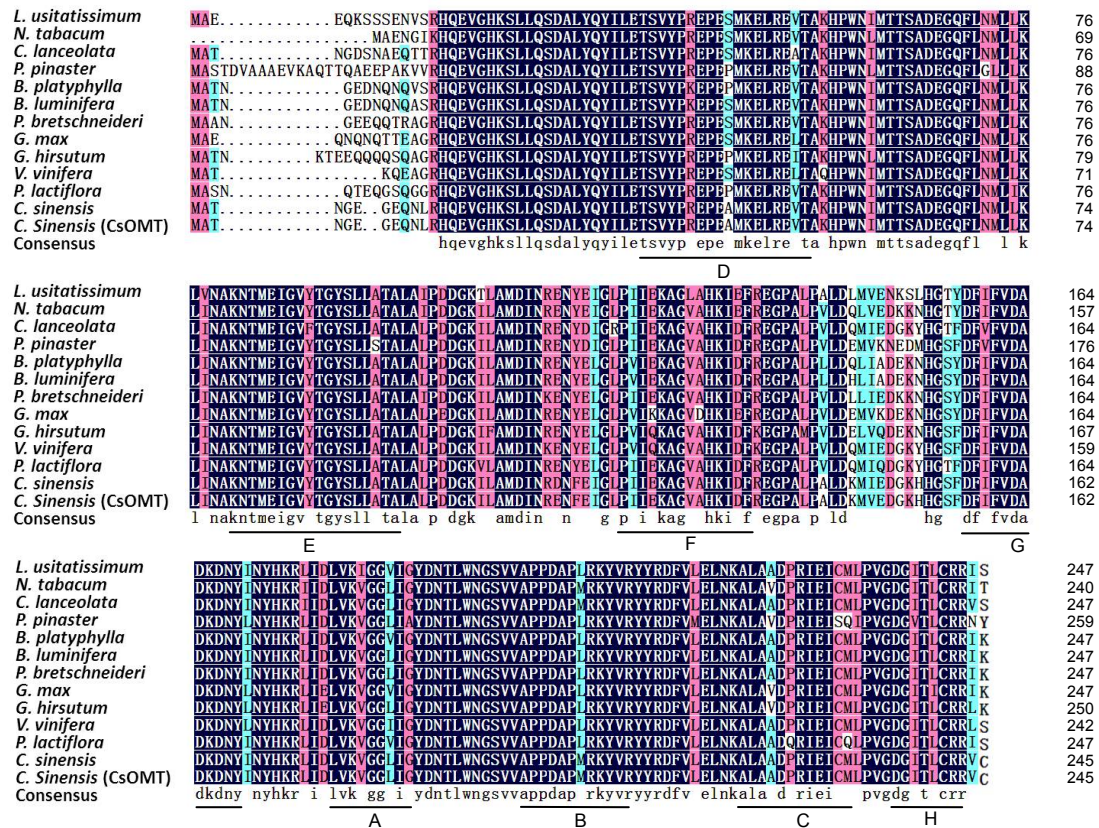
Fig. 2 Amino acid sequence alignment of CsCCoAOMT, CCoAOMT proteins from other plant species, and CsOMT from *C. sinensis*

Three putative SAM-binding motifs (A, B, and C) and additional CCoAOMT signature motifs (D, E, F, G, and H) are labeled. The sequences are from *Codonopsis lanceolata* (AB243012.1), *Betula platyphylla* (AY860952.1), *Betula luminifera* (FJ410449.1), *Vitis vinifera* (XM_002285070.1), *Pyrus bretschneideri* (JX457336.1), *Linum usitatissimum* (EU926495.1), *Paeonia lactiflora* (JQ684014.1), *Glycine max* (XM_003518654.1), *Nicotiana tabacum* (AF022775.1), *Gossypium hirsutum* (FJ848871.1), *Pinus pinaster* (FN824798.2), and *Camellia sinensis* (JQ790527). CsOMT is from *Camellia sinensis* (DD361102)

Fig. 3 Analysis of CsCCoAOMT expression and purification

(a) Analysis of prokaryotic expression of CsCCoAOMT by SDS-PAGE. 1, marker; 2, induction without IPTG; 3, total protein induced with IPTG for 16 h; 4, soluble protein in the supernatant induced with IPTG for 16 h; 5, insoluble inclusion body in sediment induced with IPTG for 16 h. (b) Analysis of purified CsCCoAOMT by SDS-PAGE. 1, marker; 2 and 3, soluble protein of CsCCoAOMT without purification (as control); 4 and 5, soluble protein of CsCCoAOMT purified in 200 mmol/L imidazole eluant
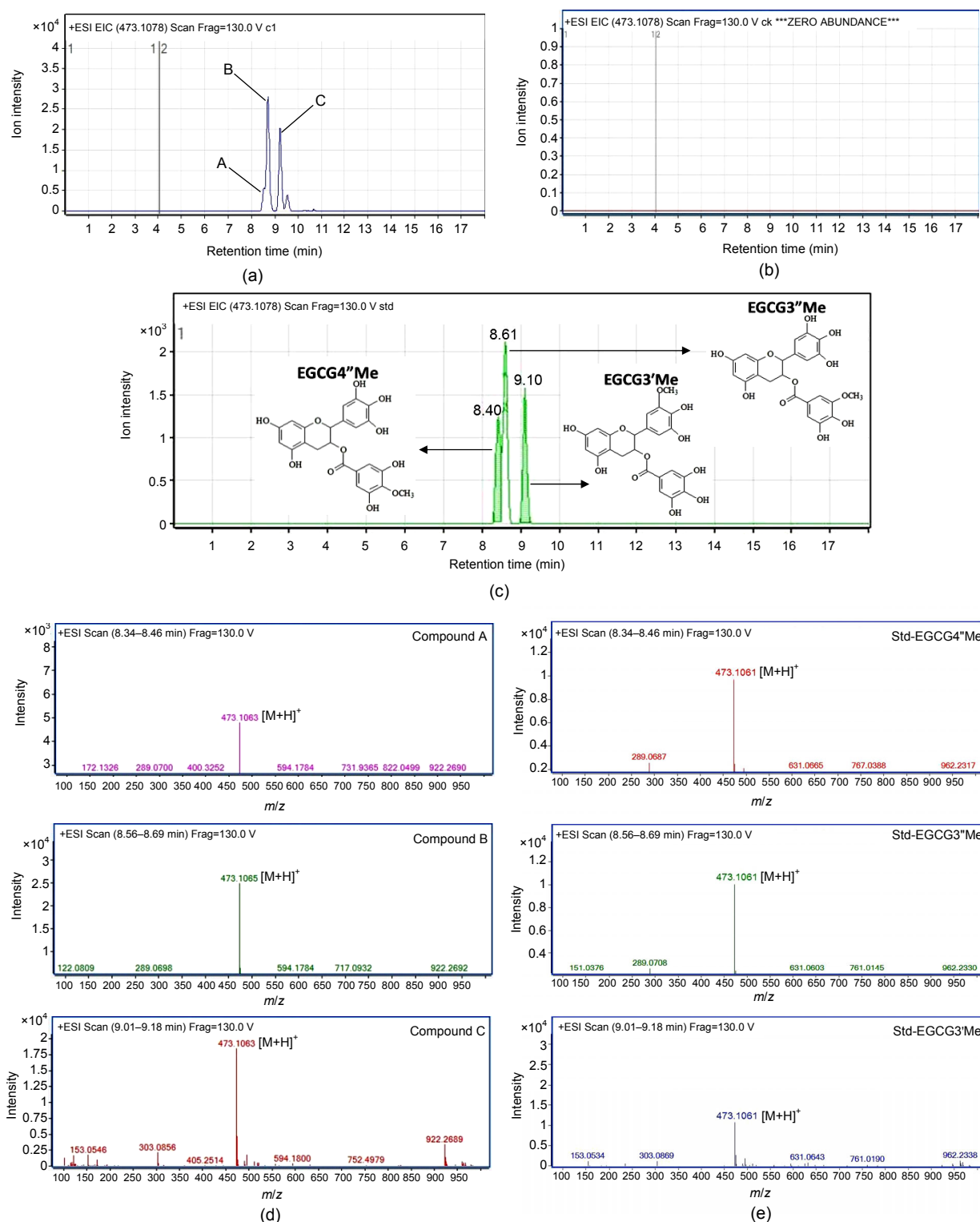
Fig. 4 Ion chromatogram and MS patterns for methylated products catalyzed by CsCCoAOMT and the monomethyl-ated EGCG standards

(a) Ion chromatogram of monomethylated products catalyzed by CsCCoAOMT. (b) Ion chromatogram of control catalyzed by heated CsCCoAOMT. (c) Ion chromatogram for EGCG3"Me, EGCG4"Me, and EGCG3'Me standards. (d) MS patterns for compounds A, B, and C in (a). (e) MS patterns for the standards EGCG4"Me, EGCG3"Me, and EGCG3'Me. The LC-MS conditions are described in Section 2.5. The deviation of *m/z* was less than 5×10^{-6}

The kinetic parameters of CsCCoAOMT were determined by reaction with EGCG as substrate, with SAM as the methyl donor (Table 1). CsCCoAOMT catalyzed the formations of EGCG3"Me, EGCG4"Me, and EGCG3'Me. All the three monomethylated EGCGs are used as products for determining the kinetic parameters. An apparent K_m value of 101 µmol/L and an apparent K_{cat} value of 0.184 s^{-1} were measured for CsCCoAOMT with EGCG concentrations ranging from 50 to 500 µmol/L. In reactions of CsCCoAOMT with the methyl donor SAM at concentrations ranging from 80 to 400 µmol/L, CsCCoAOMT catalyzed the formation of methylated EGCG with an apparent K_m value of 160 µmol/L and an apparent K_{cat} value of 0.049 s^{-1}. The presented values are the averages of duplicate experiments.

**Table 1   Kinetic parameters of CsCCoAOMT with EGCG as substrate**

Substrate/ methyl donor	V_{max} (µmol/(mg·s))	K_m (µmol/L)	K_{cat} (s^{-1})	K_{cat}/K_m (L/(mmol·s))
EGCG	0.29±0.02	101±7.9	0.184±0.02	1.84
SAM	0.24±0.05	160±18	0.049±0.007	0.31

Data are expressed as mean±standard deviation (SD). K_m, which is commonly known as Michaelis constant, is defined as the substrate concentration when the reaction velocity reaches half of the maximum (V_{max}). K_{cat} expresses the number of substrate molecules turned over per enzyme molecule (or per activity center) per second; thus, K_{cat} is sometimes called the turnover number

3.5 Analysis of gene expression by RT-PCR

Four tea cultivars (T1, T2, T3, and T4) that differing greatly in EGCG3"Me contents were selected to analyze the relative gene expression by RT-PCR. The mean contents of EGCG3"Me detected by HPLC in the four cultivars were 1.23% (T1), 0.82% (T2), 0.63% (T3), and 0.12% (T4), respectively. Semi-quantitative expression analysis of *CsCCoAOMT* indicated that the gene was expressed in all of the cultivars. The relative expression level was high in the cultivar with the highest content of EGCG3"Me (T1), and intermediate in cultivars that accumulate moderate amounts of EGCG3"Me (T2 and T3), whereas in T4 EGCG3"Me was almost undetectable by HPLC and the relative expression level of *CsCCoAOMT* was the lowest of the four cultivars (Fig. 5). Therefore, the relative gene expression levels in the four tea cultivars showed a tendency to increase consistent with increasing EGCG3"Me content in fresh leaves.
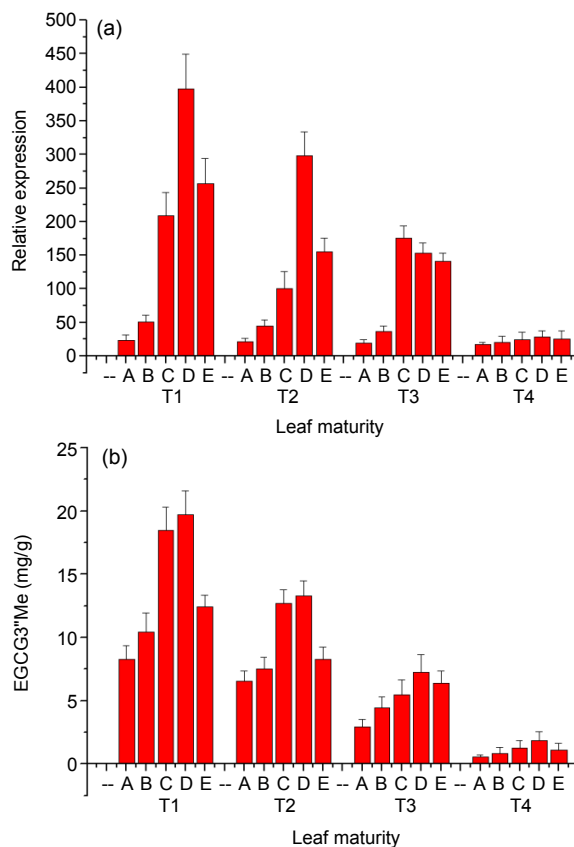
Fig. 5   Analysis of gene relative expression and EGCG3"Me content in leaves of four tea cultivars (T1, T2, T3, and T4)

A–D are different stages of leaf maturity: A, the first leaf and a bud; B, the second leaf; C, the third leaf; D, the fourth leaf; E: the mixed leaves above. (a) Relative expression of *CsCCoAOMT* quantified by RT-PCR; (b) EGCG3"Me content in leaves detected by HPLC

The relative expression level of *CsCCoAOMT* was further evaluated at different stages of leaf maturity (the first leaf and bud, the second leaf, the third leaf, and the fourth leaf from the shoot apex) in the four cultivars. The RT-PCR analysis demonstrated that rapid increase in transcript accumulation at the most advanced stages of maturity (the third leaf or fourth leaf), compared with the earliest stage (the first leaf and bud), indicating that the expression level of *CsCCoAOMT* was enhanced with increasing leaf maturity (Fig. 5a). There was a positive correlation between leaf maturity and EGCG3"Me content detected by HPLC (Fig. 5b), and with expression of *CsCCoAOMT*. These results indicated that *CsCCoAOMT* may be associated with biosynthesis of methylated catechin in *C. sinensis*.

4 Discussion

In this study, genomic and cDNA clones for the *CsCCoAOMT* gene were isolated from *C. sinensis.* This is the first report of isolation of a genomic DNA clone for *C. sinensis CCoAOMT*. In addition, we obtained multiple *CsCCoAOMT* cDNA sequences (data not shown) that encoded four deduced amino acid sequences, in which a few differences were observed at several regular sites, but the homogeneity of the sequences was more than 99%. The genetic background of *C. sinensis* is highly complex, so the reasons why there are sequence differences need further study. Moreover, the nucleotide and deduced amino acid sequences of CsCCoAOMT showed high homology with CCoAOMTs from 11 other plant species, which demonstrated that the gene is highly conserved among species and that the clone effectively codes for CCoAOMT. The alignment of the corresponding protein sequences from 11 other plant species (Fig. 2) showed 87%–92% homology with the *C. sinensis*-predicted protein sequence. The proposed structure for CsCCoAOMT consists of three putative SAM-binding motifs (A, B, and C) and five other regions (D, E, F, G, and H) that may serve as CCoAOMT signatures in plants. Accordingly, the CsCCoAOMT amino acid sequence predicted here revealed the presence of the eight mentioned motifs (A–H) with conserved spatial relationships (Fig. 2).

As a methyl donor, SAM plays an important role in the formation of methylated EGCGs. We presumed that the number and type of methylated EGCG derivatives in the reaction mixture were related to the concentration of SAM. With increasing concentration of SAM, the catalysate became more complex and EGCG derivatives with multiple methyl groups were detected. It is reported that increasing the number of methyl groups of EGCG, especially in the galloyl moiety, helps it to acquire lipophilicity and show improved absorption and stability in animal and human plasma (Kirita *et al.*, 2010). Therefore, we believe that the present study contributes effectively to the preparation of methylated EGCG. However, as is well known, CCoAOMT is a key enzyme in lignin biosynthesis and particularly in guaiacyl unit production. In the present experiments, CsCCoAOMT activity was not as high as we expected for catalyzing the synthesis of methylated EGCG. Therefore, a search for an EGCG-OMT that exhibits higher efficiency is required for efficient synthesis of EGCG3"Me.

Compliance with ethics guidelines

Yue ZHANG, Hai-peng LV, Cheng-ying MA, Li GUO, Jun-feng TAN, Qun-hua PENG, and Zhi LIN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：茶树中咖啡酰辅酶 A 氧甲基转移酶的基因克隆及其催化活性研究

目　的：从茶树中克隆一种可以催化表没食子儿茶素没食子酸酯（EGCG）生成甲基化 EGCG 的酶——咖啡酰辅酶 A 氧甲基转移酶（CCoAOMT），实现甲基化 EGCG 的酶学合成，为甲基化 EGCG 的进一步开发利用提供理论依据和技术指导。

创新点：本研究首次从茶树中克隆了一条 *CCoAOMT* 基因组序列；分析了 *CCoAOMT* 基因在不同茶树品种和不同成熟度茶鲜叶中的基因表达规律；证明了 CCoAOMT 具有催化合成甲基化 EGCG 的生物活性。

方　法：采用聚合酶链式反应（PCR）和序列分析获得 *CCoAOMT* 的编码序列和基因组序列；采用高效液相色谱-四级杆-飞行时间串联质谱技术（HPLC-QTOF-MS）分析酶促反应生成的甲基化 EGCG 产物（图4）；采用实时荧光定量 PCR 分析 *CCoAOMT* 基因的表达差异（图5）。

结　论：本研究从茶树中克隆了 *CCoAOMT* 基因的编码序列（738 bp）和基因组序列（2678 bp），明确了该基因具有 4 个内含子和 5 个外显子；揭示了 CCoAOMT 可以催化 EGCG 生成 EGCG4"Me、EGCG3"Me 和 EGCG3'Me 等多种甲基化产物；证明了 CCoAOMT 具有催化生成甲基化 EGCG 的活性；并发现该基因的表达量高低与茶鲜叶的成熟度呈正相关关系。

关键词：茶树；咖啡酰辅酶 A 氧甲基转移酶（CCoAOMT）；催化活性；甲基化表没食子儿茶素没食子酸酯（EGCG）