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Research paper

Characterization of a caffeic acid 3-*O*-methyltransferase from wheat and its function in lignin biosynthesis

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Abstract

Caffeic acid 3-*O*-methyltransferase (COMT) catalyzes the multi-step methylation reactions of hydroxylated monomeric lignin precursors, and is believed to occupy a pivotal position in the lignin biosynthetic pathway. A cDNA (*TaCM*) was identified from wheat and it was found to be expressed constitutively in stem, leaf and root tissues. The deduced amino acid sequence of *TaCM* showed a high degree of identity with COMT from other plants, particularly in SAM binding motif and the residues responsible for catalytic and substrate specificity. The predicted TaCM three-dimensional structure is very similar with a COMT from alfalfa (MsCOMT), and TaCM protein had high immunoreactive activity with MsCOMT antibody. Kinetic analysis indicated that the recombinant TaCM protein exhibited the highest catalyzing efficiency towards caffeoyl aldehyde and 5-hydroxyconiferaldehyde as substrates, suggesting a pathway leads to S lignin via aldehyde precursors. Authority of *TaCM* encoding a COMT was confirmed by the expression of antisense *TaCM* gene in transgenic tobacco which specifically down-regulated the COMT enzyme activity. Lignin analysis showed that the reduction in COMT activity resulted in a marginal decrease in lignin content but sharp reduction in the syringl lignin. Furthermore, the TaCM protein exhibited a strong activity towards ester precursors including caffeoyl-CoA and 5-hydroxyferuloyl-CoA. Our results demonstrate that TaCM is a typical COMT involved in lignin biosynthesis. It also supports the notion, in agreement with a structural analysis, that COMT has a broad substrate preference.

Keywords: *Triticum aestivum* L.; Caffeic acid 3-*O*-methyltransferase; 5-Hydroxyferulic acid *O*-methyltransferase; Caffeoyl CoA 3-*O*-methyltranferase; Lignin biosynthesis

1. Introduction

Lignin is a phenolic cell wall polymer covalently linked to the cellulose and hemicellulose components of the plant cell wall. The presence of lignin is exclusively confined to vascular plants and implies an important role for lignin in the successful colonization of land by plants. Lignin is mainly deposited in the walls of certain specialized cells such as the tracheary elements, sclerenchyma and phloem fibers. It imparts rigidity and structural support to the wall and assists in the transport of water and nutrients within xylem tissue by decreasing the permeability of the cell wall. In addition, lignification of plant tissue also occurs in response to various environmental cues such as mechanical stress or pathogen attacks [1].

The biochemical pathway for the lignin subunit (monolignol) formation consists of successive hydroxylation and *O*-methylation of the aromatic ring and conversion of the side-chain carboxyl to an alcohol function. The three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, form a complex, three-dimensional polymer of lignin by successive dehydrogenative polymerization reactions. The coniferyl and sinapyl alcohols have methyl groups that are methylated in *S*-adenosylmethionine (SAM)-dependent reactions. Incorporation of an increased proportion of coniferyl alcohol into the composition of lignan will increase the number of beta-5 linked dimers in the structure and therefore influence the structural integrity of the lignin. Thus, methylation of

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hydroxylated monomeric lignin precursors represents a critical step in lignin biosynthesis that influences the final lignin composition and structure. The monomeric composition of lignin varies with the phylogenetic origin, tissue type, stage of plant development and subcellular location [2]. Furthermore, lignin composition also affects the economics of wood pulp production [3] and forage digestibility [4]. Therefore, the enzymes for monolignol methylation are believed to occupy a pivotal position in the lignin biosynthesis pathway as well as in genetic engineering to modify lignin content and composition.

It is believed that two structurally distinct methyltransferases (OMT) are responsible for the methylation of lignin precursors. The caffeic acid 3-*O*-methyltransferase (COMT, EC 2.1.1.68) methylates caffeic acid and 5-hydroxyferulic acid [5,6], whereas caffeoyl CoA 3-*O*-methyltranferase (CCoMT, EC 2.1.1.104) methylates caffeoyl-CoA and 5-hydroxyferuloyl-CoA [7,8]. In dicotyledonous angiosperms, both COMT and CCoMT are bi-specific. In contrast, COMT in gymnosperms is believed to catalyze caffeic acid and not 5-hydroxyferulic acid. However, the substrate specificities of the OMT in monocotyledonous angiosperms are much less explored. Recently, a novel multifunctional OMT was found in loblolly pine which catalyzes the methylation of lignin precursors both at the free acid and the CoA ester levels [9]. Alfalfa COMT (MsCOMT) exhibits the higher catalyzing efficiency towards 5-hydroxyconiferaldehyde and caffeoyl aldehyde rather than caffeic acid [10]. Clearly, the full repertoire of methylation reactions involved in monolignol biosynthesis is not fully understood. We are particularly interested in the lignin biosynthesis in wheat, because of its agricultural importance and biological significance. Here, we report the biochemical analysis of a COMT from wheat and its function in lignin biosynthesis in transgenic tobacco.

2. Materials and methods

2.1. Plant materials and nucleotide acid isolation

Wheat (*Triticum aestivum* L. cv. H4564) plants were grown in a naturally lit glasshouse. Total RNA was isolated from wheat stem, root and leaf tissues using the TRI reagent (Molecular Research Center, Inc, Cincinnati, USA) and following the manufacturer's instructions. Poly(A)$^+$ RNA was isolated using PolyAT tract® mRNA Isolation Kit (Promega). RT–PCR reactions were carried out and a specific probe for COMT was obtained [11]. Genomic DNA was purified from young leaf tissues according to the protocol described by Dellaporta et al. [12]. A wheat stem cDNA library was constructed following the manufacturer's instructions (Stratagene). cDNA library screening was according to the procedure of Ma [13]. cDNA inserts were sequenced using an ABI 377 automated DNA sequencing machine following established protocols. Sequence similarities were analyzed using the SIM-Alignment Tool [14] and the data from GenBank database. Evolutionary relationships were determined using the ClustalW method with PAM 250 residue weight table [15].

2.2. Secondary and three-dimensional structure prediction

Secondary and three-dimensional structures were predicted using LOOPP software by submitting the sequence to the prediction PHD server at Cornell University (http://ser-loopp.tc.cornell.edu/cbsu/loopp.htm) and the three-dimensional figure was prepared with the program MOLMOL (http://www.mol.biol.ethz.ch/wuthrich/software/molmol) [16].

2.3. RNA gel blot analysis

Ten micrograms of total RNA was electrophoresed on 1.4% (w/v) formaldehyde agarose gels. RNA was blotted onto Hybond-N$^+$ membrane (Amersham) using established protocols [17]. The blot was hybridized at 42 °C in 6× SSC, 5× Denhardt's, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA with 50% formamide and washed with 0.1× SSC plus 0.1% SDS at 65 °C. Probes were ^{32}P-labeled using a Ready-to-Go DNA Labeling Kit (Amersham). RNA hybridization signals were normalized by a soybean 18S ribosomal RNA [18].

2.4. Expression and purification of TaCM protein in Escherichia coli

For the convenient cloning of the *TaCM* cDNA in the pET-28a vector (Novagen, USA), the NdeI and EcoRI sites were introduced by PCR at the ATG start codon and TAG stop codon, respectively. PCR reactions were carried out with 1 μmol/L primers, 0.4 mmol/L of each dNTP and 2.5 U Taq DNA polymerase (Gibco) using 5′-primer as 5′-GGAATTCCA TATGGGGTCGATCGCCGCCGG-3′, and 3′-primer as 5′-CGGAATTCCTACTTAGTGAACTCGATGG-3′. The fidelity of the PCR amplification was confirmed by DNA sequencing. The PCR product was digested with NdeI and EcoRI and ligated into pET-28a vector, and then introduced into *Escherichia coli* strain BL21 cells. Purification of recombinant TaCM protein was according to the procedure of Ma and Tian [19]. Briefly, the induced *E. coli* cells were pelleted and resuspended in extraction buffer then sonicated with lysozyme. The supernatant was further purification using Ni-NTA His-Bind® Resin (Novagen) according to the manufacturer's instructions. All purification steps were carried out at 4 °C and the purified enzyme was kept in 4 °C which was stable for 1 week.

2.5. Enzyme extraction, activity and kinetic assay

Enzyme extraction from plant tissues was according to the method of Pincon et al. [20]. COMT and CCoMT enzyme activities were measured according to the method of Ni et al. [21]. A non-induced *E. coli* extract was used as a control. Protein concentrations were determined by the Bradford assay [22] with BSA as standard. Caffeic acid was obtained from Sigma–Aldrich (Beijing, China). Caffeoyl-CoA and 5-hydroxyferuloyl-CoA were synthesized according to Stockigt et al. [23]. Caffeoyl aldehyde, 5-hydroxyconiferaldehyde, caffeoyl alcohol and 5-hydroxyconiferyl alcohol were

synthesized according to Chen et al. [24]. K_m and V_{max} values were determined by extrapolation from Lineweaver–Burk plots. The enzymes from different preparations gave the similar kinetic data therefore one typical data set from a single recombinant enzyme preparation was used for final calculations.

2.6. Protein gel blot analysis

Protein samples were resolved by 12% SDS–PAGE, then electrotransferred onto nitrocellulose membrane. Western blot analysis with rabbit anti-alfalfa COMT serum was according to the method of Kersey et al. [25].

2.7. Generation of COMT down-regulated transgenic tobacco

The *TaCM* cDNA fragment in pBluescript plasmid was cut out by SmaI and KpnI and subcloned into plasmid pGEM-7Zf(+) in the same enzyme sites. This plasmid was cut by XbaI and SacI and the isolated fragment was then ligated into the same sites of the binary vector pBI121, creating the antisense expression construct. The resulting binary vector was transferred by the freeze-thaw method into *Agrobacterium tumefaciens* strain LBA4404. Tobacco (*Nicotiana tabacum* cv Wisconsin 38) was transformed by the leaf disc method as previous described [26]. Rooted transformants were transferred to soil, grown in the greenhouse and allowed to self-pollinate. Total DNA was isolated from young leaf tissue of each tobacco lines and PCR reactions were conducted with 5′-primer: 5′-AAGGTCCTCATGGAGAGCTG-3′, and 3′-primer: 5′-CGG CAGGATGCATCCACGGA-3′, which corresponded to wheat *TaCM* cDNA sequence. The temperature program for PCR was 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 56 °C, and 1 min 30 s at 72 °C, followed by 10 min at 72 °C. The amplified products were resolved on a 0.8% agarose gel and then photographed.

2.8. Lignin analysis

Lignin content was quantitatively measured by using the Klason method [27]. The Klason lignin was expressed as a percentage of the cell wall residue (CWR). Tobacco stems were free-hand sectioned with a razor blade, and sections were stained for lignin with phloroglucinol–HCl (Wiesner reaction) [28] or potassium permanganate (Mäule reaction) [29]. Sections were observed under a dissection microscope by using dark-field illumination.

All quantitative data had at least three replications. Probability value was estimated by the Student's *t*-test at $P_{0.05}$ and $P_{0.01}$ levels.

3. Results

3.1. Characterization of TaCM cDNA in wheat

RT–PCR with degenerate primers was used to amplify COMT sequences from wheat mRNA [11]. Using PCR-produced cDNA fragment as a probe, four positively hybridizing plaques were detected after high-stringency hybridization of a wheat stem cDNA library. Restriction analysis showed that all of the isolates had the same restriction fragment pattern. Complete DNA sequence analysis of these positive clones showed that they belonged to the same cDNA, designated as *TaCM* (GenBank accession no. EF413031).

The insert of *TaCM* is 1344 nucleotides in length with a single ORF of 1071 nucleotide flanked by 5′- and 3′-untranslated region of 75 and 198 nucleotides respectively. The ORF encodes a predicted protein of 356 amino acids with a relative molecular mass of 38,600. *TaCM* was found to be almost identical to a sequence from wheat named *TaOMT2* (GenBank accession no. DQ223971) [30]. Only one amino acid difference was found between the two proteins, but *TaCM* had a 100 bp longer 3′UTR.

TaCM showed a high degree of similarity with published COMT sequences both at the nucleotide and amino acid levels. The phylogenetic reconstruction using TaCM and sequence data from GenBank showed that TaCM and all other COMT proteins clustered to one group. All CCoMT proteins clustered to another group. AEOMT from the loblolly pine diverged from both COMT and CCoMT (Fig. 1). The TaCM amino acid identity was only about 12% of CCoMT and 30% of AEOMT, while it had 75% identity with other COMT proteins.

Sequence alignment of TaCM with other typical COMT proteins from various plants showed a high degree of similarity in both SAM binding motif and the residues responsible for catalytic and substrate specificity (Fig. 2). For instance, nine amino acid residues involved in the SAM binding site have only one amino acid difference between TaCM (LVDVGGGVG) and MsCOMT (LVDVGGGTG). Particularly, TaCM has the same substrate binding and positioning residues (residues of M^{130}, N^{131}, L^{136}, A^{162}, H^{166}, F^{172}, F^{176}, M^{180}, H^{183}, I^{319}, M^{320}, N^{324}) as MsCOMT except for one amino acid difference, with V instead of I in the position 316 [6]. Furthermore, the catalytic residues (H^{269}, E^{297} and E^{329}) in MsCOMT are the same as those in TaCM.

The secondary structure of the putative TaCM protein was composed of 46.9% alpha helix, 17.7% beta sheet, 14.3% bend, 11.5% random coil and 9.6% beta turn. Alpha helices and beta sheets were the basic elements of the TaCM structure, which were interlaced with random coils, bends and turns (Fig. 3). The three-dimensional structure of TaCM is very similar to MsCOMT that has been analyzed by crystallography [6], including the SAM binding motif and the reaction active site (Fig. 3).

TaCM mRNA was detected in leaves, stems and roots, at similar levels, indicating *TaCM* was associated with constitutive lignification process in various vegetable growing tissues (data not shown). This expression pattern is different from that of other monocot *COMT* genes. For example, *COMT* from maize is highly expressed only in root [31], while *COMT* from ryegrass is abundantly expressed in the stem [32].

3.2. Heterologous expression and purification of recombinant TaCM protein in E. coli

The distribution of recombinant TaCM protein in the BL21 (DE3) strain was tested at different inducing

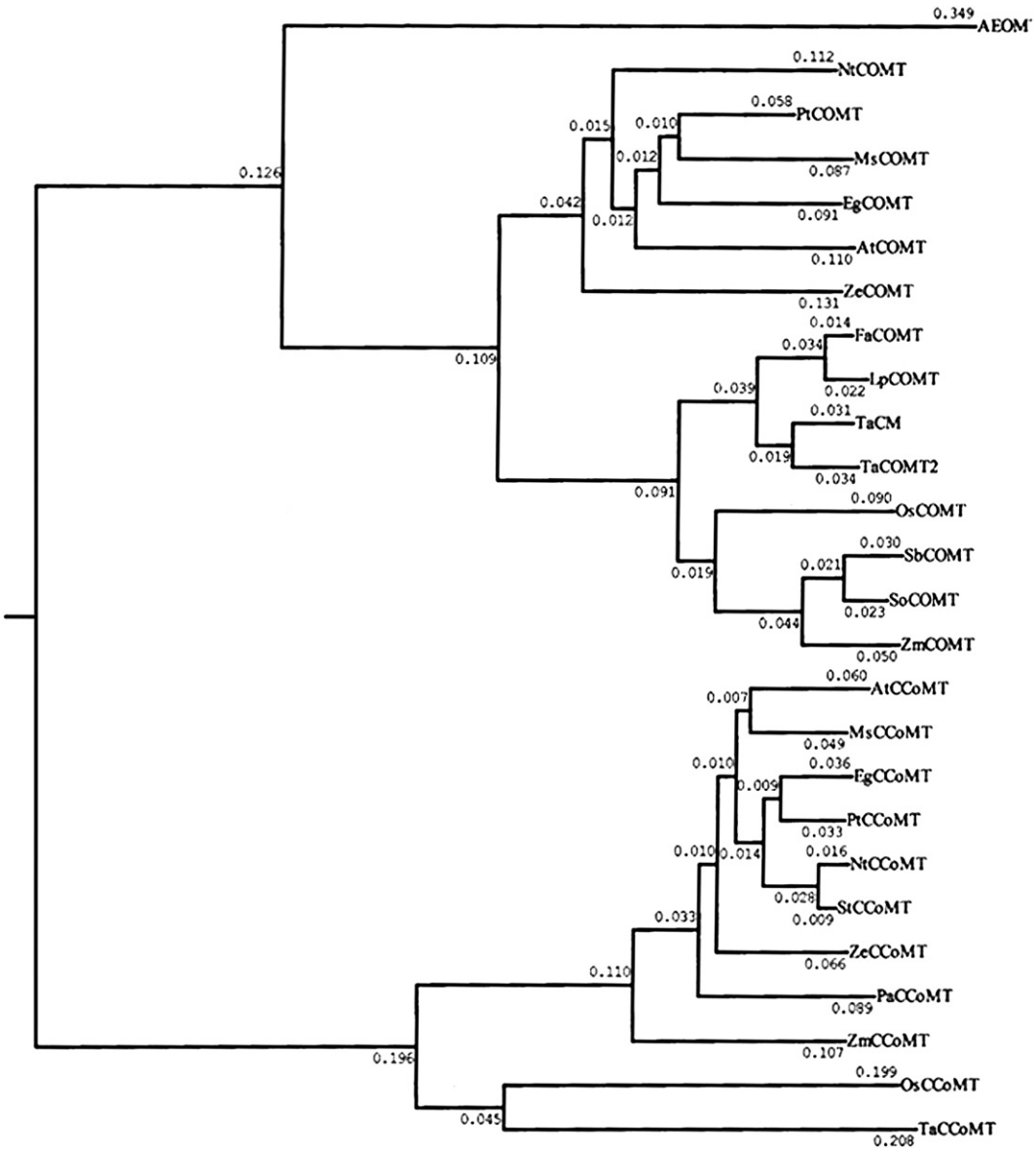


Fig. 1. Dendrogram showing phylogenetic relationships of COMT and CCoMT proteins from various plants. The tree was constructed using the ClustalW method with PAM 250 residue weight table and boot-strap values were shown in each branch. AtCOMT (GenBank accession no. AB012287) and AtCCoMT (AL021961) from *Arabidopsis thaliana*; EgCOMT (X74814) and EgCCoMT (Y12228) from *Eucalyptus gunnii*; FaCOMT (AF153825) from *Festuca arundinacea*; LpCOMT (AF010191) from *Lolium perenne*; MsCOMT (M63853) and MsCCoMT(U20736) from *Medicago sativa*; NtCOMT (X74452) and NtCCoMT (U62734) from *Nicotiana tabacum*; AEOMT (U39301) and PaCCoMT (AF036095) from *Pinus taeda*; PtCOMT (U13176) and PtCCoMT (U276116) from *Populus treamuloid*; OsCOMT (DQ288259) and OsCCoMT (NP_001056910) from *Oryza sativa*; SoCOMT (AJ231133) from *Saccharum officinarum*; StCCoMT (AB061268) from *Solanum tuberosum*; SbCOMT (AF387790) from *Sorghum bicolor*; TaCOMT2 (AY226581) and TaCCoMT (AM050684) from *Triticum aestivum*; ZmCOMT (M73235) and ZmCCoMT (AJ242981) from *Zea mays*; ZeCOMT (U19911) and ZeCCoMT (U13151) from *Zinnia elegans*; TaCM: protein in this study (EF413031).

temperatures. An induction temperature at 16 °C increased this protein in supernatant fraction very markedly (Table 1). The recombinant TaCM protein from supernatant fraction of *E. coli* was purified to homogeneity, as determined by SDS–PAGE (Fig. 4A).

3.3. Substrate specificities and enzyme kinetic analyses of TaCM protein

The relative activities of the purified TaCM protein against potential substrates in the monolignol pathway were determined (Table 2). The lowest K_m values were for 5-hydroxyconiferaldehyde and caffeoyl aldehyde and the highest V_{max} value was for caffeoyl alcohol. From a calculation of the reaction efficiency (K_{cat}/K_m) for the TaCM protein, 5-hydroxyconiferaldehyde and caffeoyl aldehyde appeared to be the most favored substrates. The reaction efficiency of the other three substrates (caffeic acid, caffeoyl alcohol and 5-hydroxyconiferyl alcohol) were lower than 5-hydroxyconiferaldehyde but still quite reasonable.

Interestingly, the TaCM protein also accepted as substrates the CCoMT enzyme substrates, caffeoyl-CoA and 5-hydroxyferuloyl-CoA.

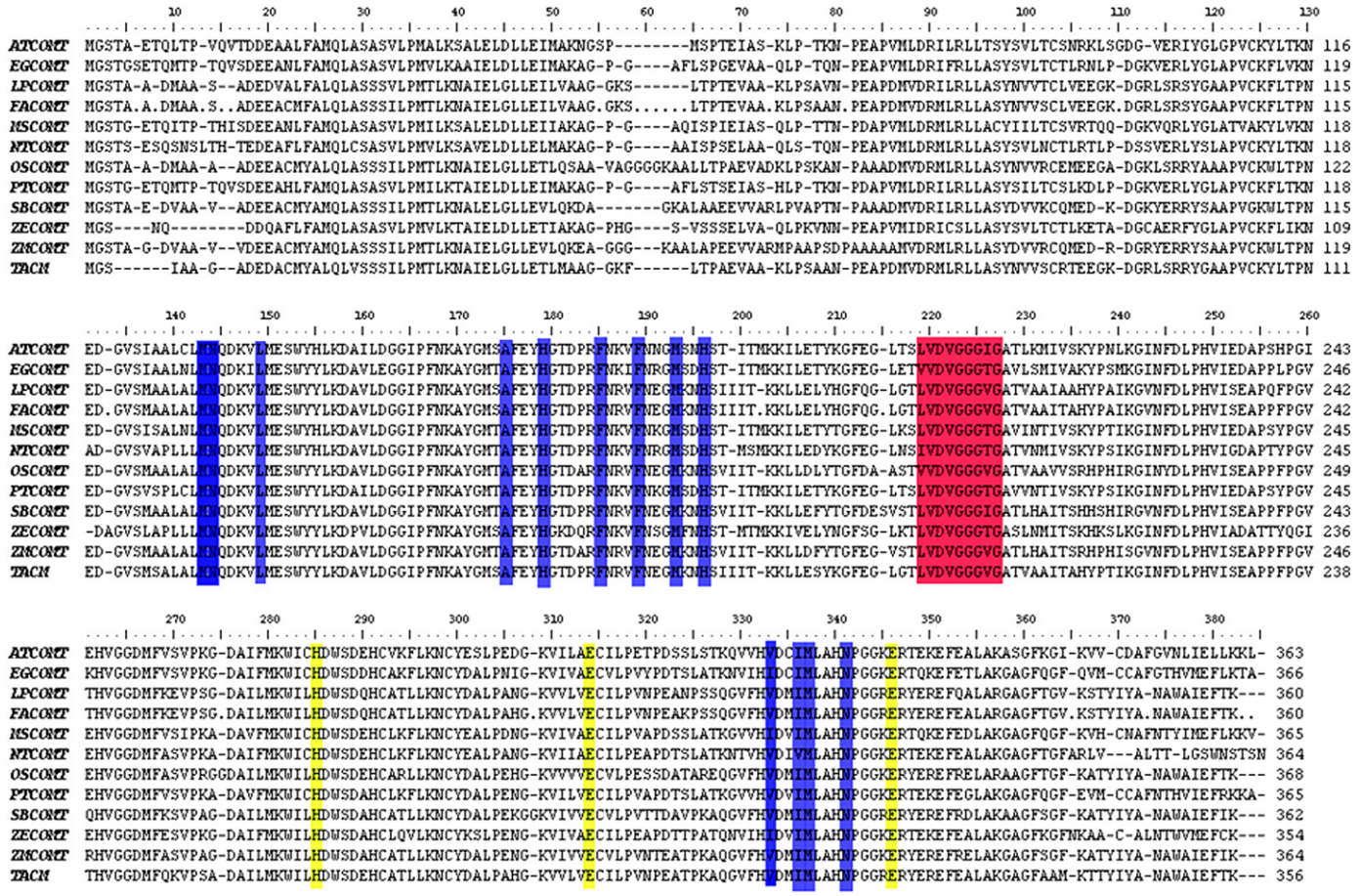


Fig. 2. Sequence alignment of TaCM and COMT from other plants. The sequence data are the same as in the Fig. 1. Conserved motif for SAM binding is labeled as red, catalytic residues are labeled as yellow, and active site substrate binding/positioning residues are labeled as blue.

3.4. Western blot analysis of recombinant TaCM protein

To further test the enzyme identity of the TaCM protein, a Western blot was conducted using rabbit anti-alfalfa COMT serum. As shown in Fig. 4B, the TaCM protein showed strong immunoreactivity towards COMT antibody.

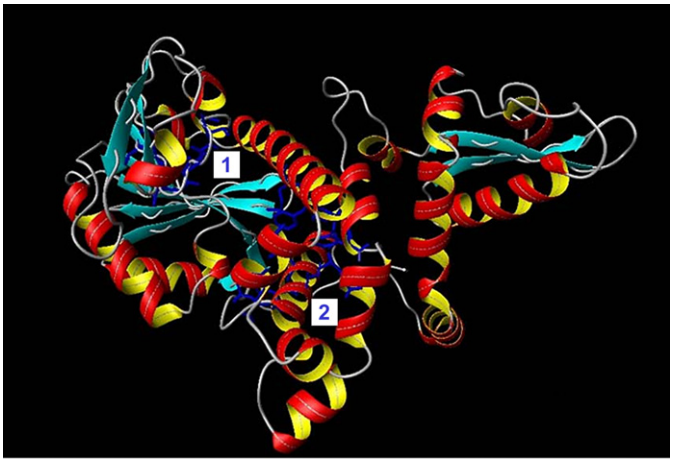


Fig. 3. The predicted three-dimensional structure of TaCM. Alpha helices are indicated in spirals, beta-sheets are indicated by arrows, and turns and coils are indicated by lines. The SAM binding motif and the active site for catalysis are indicated with the numbers 1 and 2, respectively. The data were analyzed by LOOPP software, and the figure was prepared with the program MOLMOL.

3.5. Production of antisense tobacco plants inhibited in COMT gene expression

Tobacco leaf discs were transformed using *Agrobacterium* LBA4404 containing *TaCM* in the antisense orientation relative to the 35S promoter of the binary vector pBI121. Five antisense transformants were produced which were tested for the presence of the insert gene using PCR and found to be positive (data not shown). All transgenic tobacco plants were transferred to the greenhouse and studied further.

3.6. Analysis of lignin variation in transgenic tobacco plants

Five transgenic lines containing antisense *TaCM* cassette were selected for assay of both COMT and CCoMT activities.

Table 1
Distribution of recombinant TaCM protein in pellet and supernatant fraction ($\mu g\ \mu l^{-1}$) at different inducing temperature

Temperature (°C)	Pellet	Supernatant
37	0.5	0.5
16	0.35	1.0

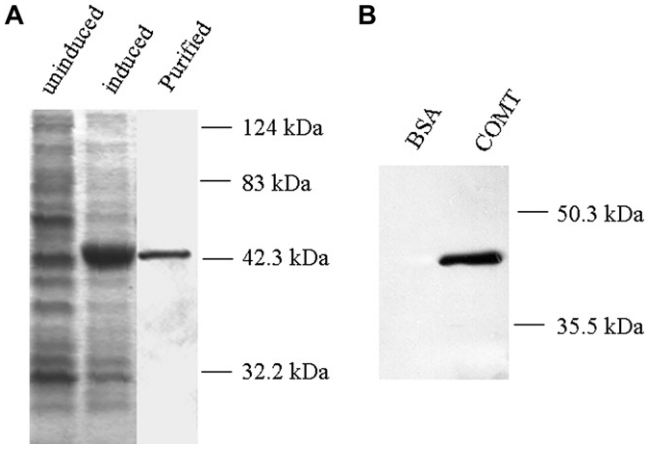


Fig. 4. Gel electrophoretic analysis of purification and protein gel blot analysis of TaCM protein expression in *E. coli*. (A) Proteins were separated from the total protein fractions of uninduced and IPTG-induced *E. coli* harboring TaCM expression plasmid, and after His-Tag resin purification (1 μg) of the soluble fraction from induced cells. (B) Recombinant TaCM protein (0.6 ng) was separated by SDS–PAGE, blotted onto nylon membranes and probed with monospecific polyclonal antisera raised against alfalfa COMT. Molecular markers are indicated on the right of the each figure.

Midstem sections (internodes 10 and 11 counting from the first fully opened leaf at the top) from 3-month-old plants grown in the greenhouse were collected for enzyme and lignin analyses. The results (Fig. 5) showed that the transgenic lines had a dramatic reduction in COMT activity (COMT activity was 32–51% of that of the wild-type tobacco which was about 0.56 μkat kg protein^{-1}). CCoMT activity, however, had little change in the transgenic lines as compared to that in the wild-type tobacco.

The Klason lignin in the transgenic lines was analyzed. The results presented in Table 3 showed that a reduction in COMT activity resulted in a marginal decrease in Klason lignin. The Klason lignin content in the transgenic lines was reduced to amounts ranging from 90.5% to 95.8% of that of the wild type. The lignin content decrease was statistically significant difference with the wild-type tobacco in only three out of five transgenic lines.

Stem sections from the transgenic and wild-type plants were subjected to Wiesner and Mäule staining (Fig. 6). Wiesner reacts with the hydroxycinnamaldehyde and benzaldehyde groups present in lignin and the color intensity generated in this reaction roughly reflects total lignin content [33].

Table 2
Kinetic parameter of TaCM protein

Substrates	V_{max} (nkat mg protein^{-1})	K_m (μM)	K_{cat}/K_m (min^{-1} μM^{-1})
Caffeic acid	2.38 ± 0.22	68.75 ± 4.21	84.74
Caffeoyl-CoA	1.26 ± 0.16	83.04 ± 5.22	37.01
5-OH-Feruloyl-CoA	1.28 ± 0.20	95.17 ± 10.1	32.85
Caffeoyl aldehyde	2.56 ± 0.29	43.72 ± 5.27	143.46
5-OH-Coniferaldehyde	1.56 ± 0.11	17.31 ± 1.56	220.51
Caffeoyl alcohol	3.55 ± 0.31	84.03 ± 6.34	103.52
5-OH-coniferyl alcohol	2.67 ± 0.23	100.21 ± 11.2	65.14

Values represent the mean of 3 independent replicates ± standard deviation.

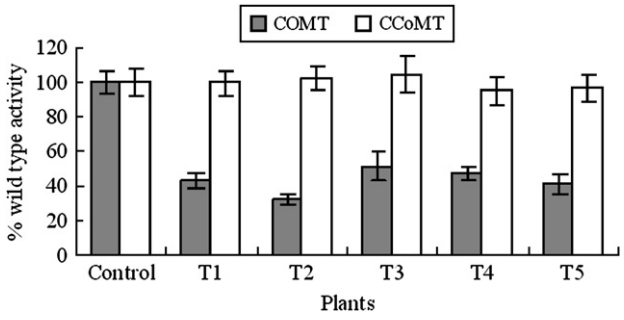


Fig. 5. COMT and CCoMT activities in transgenic tobacco plants. Three plants from each line were selected for assay of both COMT and CCoMT activities. The activities in transgenic plants were expressed as a percentage of wild-type activity. Data are the mean of 3 independent assays ± standard deviation.

Staining, which is normally bright red in control xylem, was similar in the transgenic T2 line with the wild-type tobacco (Fig. 6a,b). Maüle test, however, revealed striking variation between the wild-type and the transgenic plants. Whereas the wild type exhibited the positive purple-red coloration, the transgenic T2 displayed a brown coloration (Fig. 6c,d). Such a color shift from purple-red to brown is believed to be diagnostic for a decrease in the S units of the lignin polymer [34]. Other transgenic lines had similar results.

3.7. Growth and development of transgenic tobacco

Although antisense suppression of COMT resulted in reduction of S lignin, no significant effects on plant growth and morphology were observed when the transgenic plants were grown in the greenhouse. The transgenic plants bore normal flowers and grew to heights similar to wild-type plants. The total flavonoid contents were determined by a colorimetric assay with $AlCl_3$ [35] and showed no remarkable difference between the transgenic and wild-type tobaccos.

4. Discussion

4.1. TaCM is an authentic COMT involved in lignin biosynthesis

cDNAs for COMT and CCoMT have been cloned from several plants. In alfalfa, COMT and CCoMT have been

Table 3
Lignin content in wild-type and transgenic tobacco plants

Plants	Klason lignin (% of cell wall)	% of wild type
Wild type	19.0 ± 0.42	100
T1	17.7 ± 0.46*	93.2
T2	17.2 ± 0.39**	90.5
T3	17.9 ± 0.59	94.2
T4	18.2 ± 0.41	95.8
T5	17.5 ± 0.31**	92.1

Lignin content of the fourth internode is determined by Klason method (Kirk and Obst, 1988). Values represent the mean of 3 independent replicates ± standard deviation. Significant differences at $P_{0.05}$ and $P_{0.01}$ levels between wild-type and transgenic lines are marked by * and **, respectively.

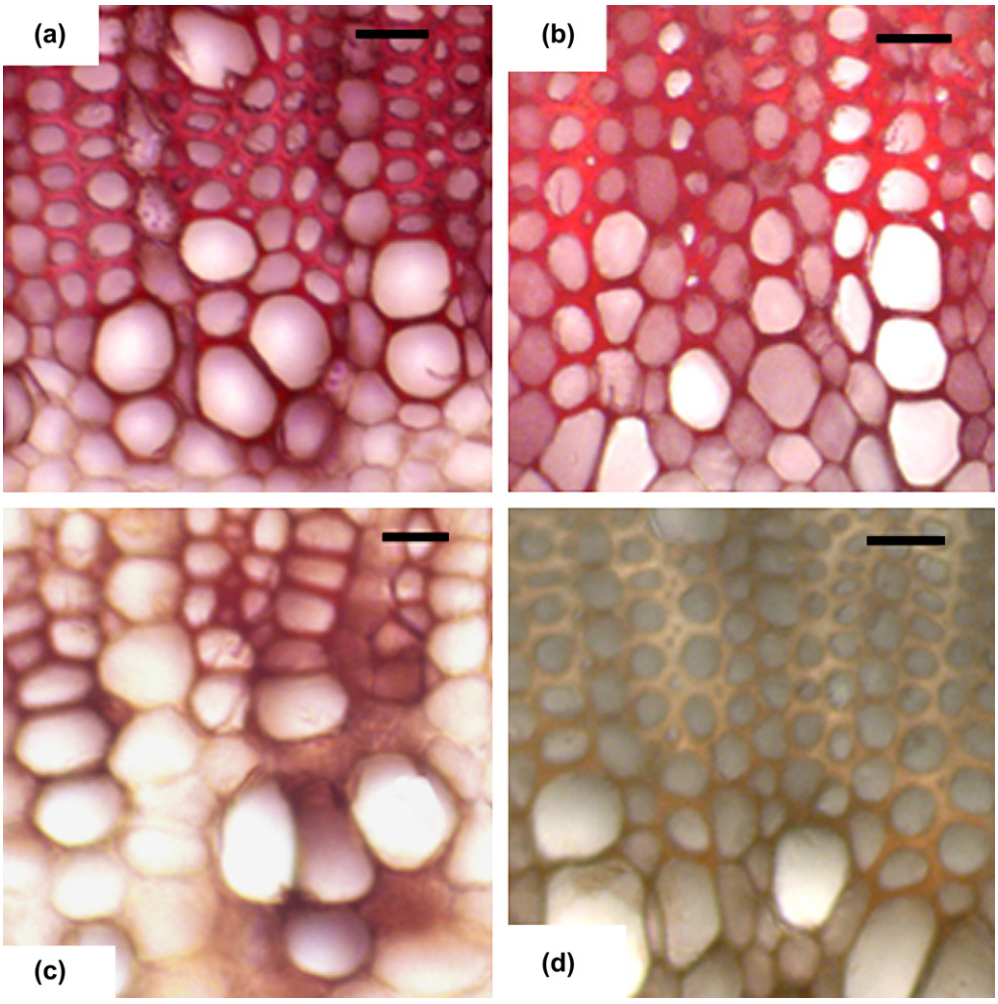


Fig. 6. Lignin staining of control and transgenic tobacco. a and b are treated with Wiesner, c and d with the Mäule reagents. a and c are control, b and d are transgenic T2 line. Bar = 80 μm.

characterized from biochemical and transgenic approaches [10,36]. Kinetic analysis of alfalfa CCoMT showed a similar K_m and V_{max} towards caffeoyl CoA and 5-hydroxyferuloyl CoA, while the COMT exhibited the highest V_{max}/K_m values with 5-hydroxyconiferaldehyde and caffeoyl aldehyde. The authors suggested that COMT but not CCoMT was essential for S lignin biosynthesis, while both COMT and CCoMT were involved in G lignin biosynthesis. In tobacco, down-regulation of COMT only decreased S units without a reduction in lignin content, and down-regulation of CCoMT resulted in a dramatic decrease in lignin content as well as reduction in both S and G units. G units were preferentially reduced in CCoMT down-regulated tobacco plants. It was therefore proposed that CCoMT plays an essential role in the synthesis of both G and S units [37,38]. Down-regulation of COMT in transgenic maize resulted in a strong decrease in lignin content, a reduction in total S units but an increase in total G units [39]. Down-regulation of COMT in transgenic tall fescue resulted in a strong decrease in lignin content and a reduction in total S and G units, but the final S/G ratio was decreased [40]. These contradictory results are possible due to unspecified differences in tissue type and maturity [2], but the differences of lignin biosynthesis and substrate preference in different plants should not be overlooked. For this reason, it is essential to analyze the methylation reactions of lignin in other plants, particularly in monocotyledonous plants.

To date, detailed biochemical analysis for COMT proteins from monocotyledonous plants is still limited. The phylogenetic reconstruction and sequence alignment of amino acid sequences showed that TaCM and all other COMT proteins clustered to one group (Fig. 1), and their proteins showed a high degree of similarity in both SAM binding motif and the residues responsible for catalytic and substrate specificity (Fig. 2). TaCM has a similar secondary structure and three-dimensional structure to MsCOMT, and showed strong immunoreactivity towards antibody for MsCOMT, a COMT protein from alfalfa that has been analyzed by crystallography [6]. These *in vitro* data suggest that TaCM belong to COMT, which may be involved in lignin biosynthesis.

The enzyme kinetic analysis indicated that TaCM exhibited a clear preference for 5-hydroxyconiferaldehyde and caffeoyl aldehyde and to a lesser extent for caffeic acid, caffeoyl alcohol and 5-hydroxyconiferyl alcohol. These results are consistent with the data from alfalfa. Although alfalfa and wheat are phylogenetically remote, these data support the hypothesis that the

pathway leading to S lignin involves the methylation of caffeoyl aldehyde and 5-hydroxyconiferaldehyde by COMT [41].

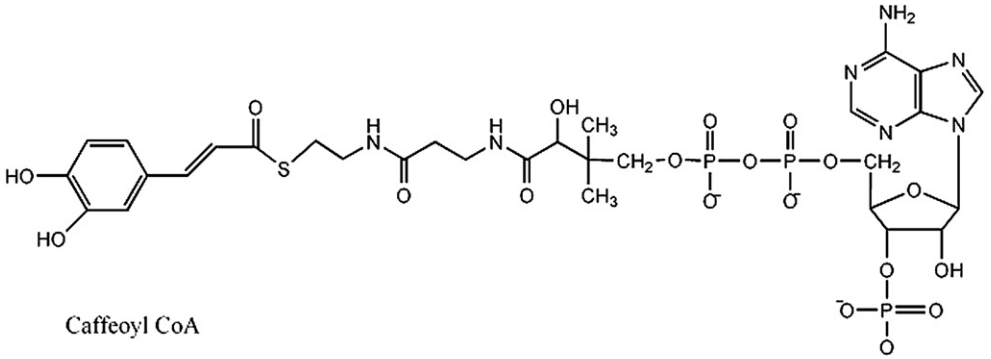
The expression of antisense *TaCM* showed a strong reduction of COMT enzyme activity in transgenic tobacco, which resulted in only a marginal decrease in lignin content but remarkable decrease in S lignin ratio. These results are consistent with the data from transgenic tobacco [38] and alfalfa [36] but different to the data from the transgenic maize [39] and tall fescue [40]. In maize and tall fescue, down-regulation of COMT led to a decrease both in Klason lignin content and S lignin ratio. Both *in vitro* and *in vivo* data suggest that TaCM is an authentic COMT involved in lignin biosynthesis, particularly in S lignin synthesis. Furthermore, TaCM may not be involved in flavonoid metabolism in normal tobacco development as the total flavonoid content is not influenced by down-regulation of TaCM. Combined with the data from tobacco, alfalfa, maize and tall fescue, our results indicate that COMT is mainly involved in the synthesis of S lignin. The roles of COMT in total lignin content and G lignin synthesis, however, are equivocal and need further investigation.

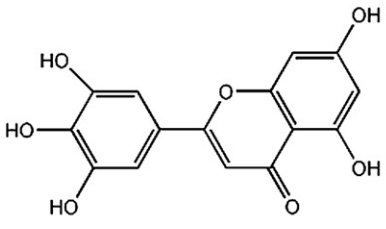
4.2. COMT enzyme may have broad substrate preferences

It is important to note that the TaCM enzyme also exhibits a strong activity towards ester precursors including caffeoyl-CoA and 5-hydroxyferuloyl-CoA. The catalytic efficiency is lower for ester precursors than that for the corresponding aldehydes. For example, the K_{cat}/K_m value for caffeoyl-CoA is 26% of that for caffeoyl aldehyde. A similar situation pertains to the AEOMT protein from loblolly pine [9]; however, the amino acid sequence of AEOMT (381 amino acids) is quite different from the COMT proteins (about 350 amino acids). TaCM protein, on the other hand, has very similar amino acid sequence and three-dimensional structure to the other COMT proteins (Figs. 2 and 3). Therefore, the activity of TaCM towards the ester precursors may be more significant.

Structural analysis of alfalfa COMT and CCoMT by crystallography has shown that the COMT active site is more spacious than for other plant OMTs such as isoflavone *O*-methyltransferase and chalcone *O*-methyltransferase [6,8], and this allows the facile accommodation of the broad range of phenolic substrates. The comparison of the CCoMT structure with other known COMT demonstrates a high degree of structural similarity in the SAM-binding region [8]. This implies a similar catalytic mechanism accompanying catalytic transfer of a methyl moiety from SAM to a suitably placed methyl acceptor between COMT and CCoMT. The TaCM protein is capable of methylating not only acid, aldehyde and alcohol precursors, but also ester precursors. This further supports the results from the structural analysis.

A recent report has identified an OMT (namely TaOMT2) from wheat as a flavonoid *O*-methyltransferase, since its main substrates were tricetin and related flavonoid compounds [30]. TaCM and TaOMT2 proteins only have one amino acid





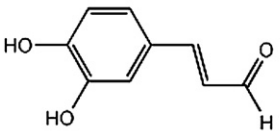


Fig. 7. The structure of proposed substrates for COMT.

difference, in which E^{89} in TaCM is replaced by D^{89} in TaOMT2. It is therefore reasonable to suggest that TaCM and related COMT can methylate phenol substrates containing aldehyde, flavonoid and CoA moieties (Fig. 7). A cDNA isolated from rice showed 73% amino acid sequence identity with TaCM (namely OsCOMT in Fig. 1). This enzyme was shown to be able to transfer the SAM methyl group to flavonoid substrates, such as eriodictyol, luteolin, quercetin and taxifolin [42], further supporting the idea that this substrate preference of COMT may be ubiquitous.

Our data indicate that COMT is not only involved in converting caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid, but also in the conversion of caffeoyl-CoA to feruloyl-CoA, and 5-hydroxyFeruloyl-CoA to sinapoyl-CoA, which has been proposed by Dixon et al. [41]. Therefore, COMT may overlap with CCoMT in some reactions. At present, the biological significance of the broad substrate preference that TaCM exhibits is unclear. One explanation may be that TaCM may have different functions during the methylation reactions of lignin and flavonoid biosynthesis in the different tissues, since the expression of *TaCM* gene is detected in almost all vegetative tissues including leaf, stem and root. Another possibility is that TaCM normally catalyzes the methylation reaction for caffeoyl aldehyde and 5-hydroconiferaldehyde but that it can use other substrates at times of mechanical stress or pathogen attack, since flavonoid compounds are generally part of the antimicrobial activity of plant defense reactions. In tomato and *Arabidopsis*, repression of lignin synthesis may lead to the redirection of the metabolic flux into flavonoids [43,44]. Tobacco COMT has reported to be induced by pathogen attaches attacks such as TMV infection. Repression of COMT or CCoMT alone in transgenic tobacco had no detectable impact on antiviral resistance but double transgenic tobacco developed larger necrotic lesions upon TMV infection [45,20]. Therefore, COMT activity towards CoA esters may promote the flavonoid production which compensates CCoMT activity in plant defense reactions. This warrants further investigation.

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