ROLES OF 5-HYDROXYCONIFERYLALDEHYDE AND CAFFEOYL COA *O*-METHYLTRANSFERASES IN MONOLIGNOL BIOSYNTHESIS IN *CARTHAMUS TINCTORIUS*

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This paper is dedicated to Professor Takayoshi Higuchi, on the occasion of his 80th birthday

Lignins and lignans are biosynthesized through the cinnamate/monolignol pathway. Maturing seeds of *Carthamus tinctorius* contain both lignins and lignans and their biosynthesis is initiated at particular stages of maturation. Therefore, this species is a good plant material for the study of lignin and lignan biosynthesis. The functions of three *C. tinctorius* caffeoyl CoA *O*-methyltransferases (CtCoAOMT1, 3 and 5) and of one *C. tinctorius* 5-hydroxyconiferaldehyde *O*-methyltransferase (CtCAldOMT1), known as key enzymes in the cinnamate/monolignol pathway, have been investigated. Enzyme kinetic and gene expression analyses proved the involvement of *CtCoAOMT3* and *CtCAldOMT1* in lignin biosynthesis.

INTRODUCTION

Lignins and lignans, representing abundant classes of phenylpropanoids, are biosynthesized from cinnamyl alcohols (monolignols), which, in turn, are biosynthesized *via* the cinnamate/ monolignol pathway and are closely related in terms of their chemical structures. Lignins are formed by non-enantioselective polymerization of monolignols, whereas typical lignans are formed by enantioselective coupling of two coniferyl alcohol units, in which the central carbon (C8) atoms of the allyl moieties are linked to each other.[1]

During plant development, lignins fill the spaces between polysaccharides in the cell wall and confer mechanical strength and imperviousness to the cell wall.[2] In defense responses, lignins get accumulated throughout the hypersensitive response region as a result of numerous plant–microbe interactions, viewed as physical barriers against pathogen infections.[3-7] On the other hand, lignans manifest a wide range of biological effects on various organisms, humans included, at molecular, enzymatic, physiological, pharmacological and even clinical levels.[8] Hence, lignans are considered to be involved in plant defense systems, although only scarce information is available on the function and role they play in the plants producing them.[9]

Considering all these, the biosynthesis of lignins and lignans has received widespread attention in relation to cell wall formation, molecular breeding of plant biomass suitable for biofuel production, plant defense and production of clinically important compounds, etc.

The cinnamate/monolignol pathway (Fig. 1) – *i.e*. the biosynthetic pathway from phenylalanine to hydroxycinnamyl alcohols – supplies precursors for various phenylpropanoid compounds, lignins and lignans included. Numerous studies devoted to the cinnamate/monolignol pathway have been conducted in relation to lignin biosynthesis. However, nothing is known on the genes encoding enzymes of the pathway for lignan biosynthesis, except for a recently proposed[10] biosynthetic route for lignan biosynthesis *via* ferulic acid.

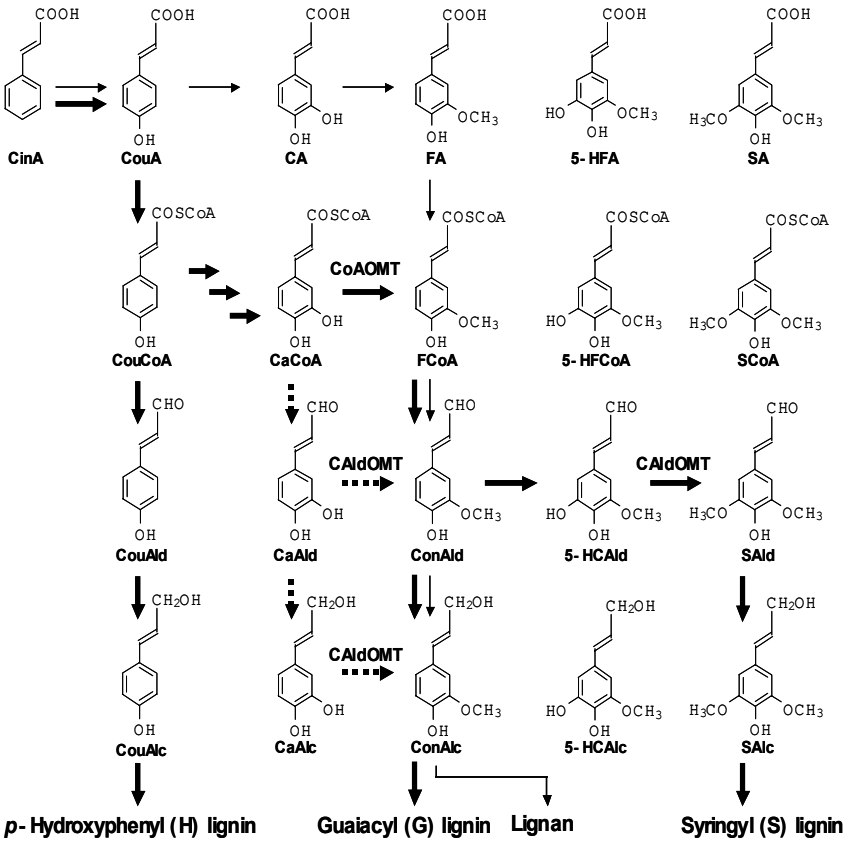

Figure 1: The cinnamate/monolignol pathway. Thick solid arrow – currently accepted lignin biosynthetic pathway; solid arrow – lignan biosynthetic pathway, as suggested by Sakakibara *et al*.;[10] dotted arrow – additional lignin biosynthetic pathway (previously described[11]); CinA – cinnamic acid; CouA – *p*-coumaric acid; CA – caffeic acid; FA – ferulic acid; 5-HFA – 5-hydroxyferulic acid; SA – sinapic acid; CouCoA – *p*-coumaroyl CoA; CaCoA – caffeoyl CoA; FCoA – feruloyl CoA; 5-HFCoA – 5-hydroxyferuloyl CoA; SCoA – sinapoyl CoA; CouAld – *p*-coumaraldehyde; CaAld – caffealdehyde; ConAld – coniferaldehyde; 5-HCAld – 5-hydroxyconiferaldehyde; SAld – sinapaldehyde; CouAlc – *p*-coumaryl alcohol; CaAlc – caffeyl alcohol; ConAlc – coniferyl alcohol; 5-HCAlc – 5-hydroxyconiferyl alcohol; SAlc – sinapyl alcohol; CoAOMT – caffeoyl CoA *O*-methyltransferase; CAldOMT – 5-hydroxyconiferaldehyde *O*-methyltransferase

Maturing seeds of *Carthamus tinctorius* (safflower) accumulate both lignins and lignans and their biosynthesis is initiated at particular moments of maturation.[10] This species is therefore a good plant material for comparative studies of lignin and lignan biosynthesis. In a first step of these comparative studies, the cDNAs encoding caffeoyl CoA *O*-methyltransferase (CoAOMT) and 5-hydroxyconiferaldehyde *O*-methyltransferase [CAldOMT, formerly caffeic acid OMT (COMT or CAOMT)], the key enzymes of the cinnamate/monolignol pathway from maturing seeds of *C. tinctorius*, were cloned, and the genes involved in lignin biosynthesis were

identified.

EXPERIMENTAL

Plant materials

Carthamus tinctorius cv. round-leaved white seeds, purchased from the Takii Seed Co. Ltd. (Kyoto, Japan), were sown in garden soil. The germinated plants were maintained after flowering and subsequent seed maturation in a plant incubator (light intensity ca. 17000 LUX, 16/8 h day/night, at 25 ºC). Immediately after harvesting, the seeds were frozen and stored in liquid nitrogen until used.

Equipment

GC-MS analysis was performed on a Shimadzu QP-5050A GC-MS system (Shimadzu) [electron impact mode (70 eV); column: Shimadzu Hicap CBP10-M25-025 (20 m × 0.22 mm); carrier gas: helium; injection temperature: 240 ºC, column temperature, 40 ºC at t_0= 2 min, then to t= 230 at 25 ºC/min]. Samples dissolved in *N*, *O*-bis(trimethylsilyl)acetoamide were subjected to GC-MS measurements after heating at 60 ºC for 45 min.

Chemicals

Caffeic acid (CA) was purchased from the Nacalai Tesque, Co., Ltd. 5-Hydroxyferulic acid (5-HFA), caffeoyl CoA (CaCoA), 5-hydroxyferuloyl CoA (5-HFCoA), caffealdehyde (CaAld), 5-hydroxyconiferaldehyde (5-HCAld), caffeyl alcohol (CaAlc), 5-hydroxyconiferyl alcohol (5-HCAlc), ferulic acid-d_3 (FA-d_3), sinapic acid-d_3 (SA-d_3), coniferaldehyde-d_3 (ConAld-d_3), sinapaldehyde-d_3 (SAld-d_3), coniferyl alcohol-d_3 (ConAlc-d_3) and sinapyl alcohol-d_3 (SAlc-d_3) were prepared as previously described.[10,11]

Molecular cloning of cDNAs encoding CtCoAOMT and CtCAldOMT

λZAP II cDNA libraries were prepared from *C. tinctorius* cv. round-leaved white seeds harvested 12 days after flowering (DAF) (T. Umezawa *et al*., manuscript in preparation) and randomly primed, ^{32}P-labelled, *Populus tremuloides CAldOMT* (GenBank accession number U13171) and *Pinus taeda CoAOMT* (GenBank accession number AF036095) cDNA probes being used to screen the cDNA libraries under low stringency conditions.

More than 2.5×10^5 plaque-forming units (PFU) of the λZAP II cDNA library (library size: 1.4×10^6 PFU/μg vector; average length: 1.9 kb) were hybridized with the conserved *S*-adenosyl-L-methionine (SAM) binding domain of *PtreCAldOMT*, under various low stringency conditions. These repeated experiments always gave a single clone which showed high homology to *PtreCAldOMT*, designated as *CtCAldOMT1*.

When the conserved SAM binding domain of *PtreCoAOMT* was hybridized with ca. 5.2×10^4 PFU of the other λZAP II cDNA library (library size: 4.6×10^6 PFU/μg vector; average length: 1.2 kb), 25 positive clones were obtained after second-round screening. Sequencing of the clones revealed that 23 clones were putative *CoAOMT*s, classified into 6 full length *CoAOMT* clones and designated as *CtCoAOMT1* – *CtCoAOMT6*. The numbers of cDNAs with sequences identical to each *CtCoAOMT* cDNA were as follows: *CtCoAOMT1* – eleven; *CtCoAOMT2* – two; *CtCoAOMT3* – six; *CtCoAOMT4* – one; *CtCoAOMT5* – two; and *CtCoAOMT6* – one.

The amino acid sequence alignment and the neighbour-joining tree were generated with the ClustalX program, while the resulting dendrograms were visualized[12] with the TREEVIEW program.[13] For the sake of comparison, the maximum likelihood method was used and it was confirmed that the profile of the resulting tree was the same as that from the neighbour-joining tree.

Expression analysis of CtCoAOMTs and CtCAldOMT

Total RNAs were extracted from maturing seeds of *C. tinctorius* and stems, by the Plant RNeasy extraction kit (Qiagen) or by the method of Bugos *et al*.[14] The concentration of isolated RNA was estimated by measuring the absorbance at 260 nm. An aliquot of the RNA extract was reverse-transcribed using Superscript II reverse transcriptase and random hexamers, according to the manufacturer's protocol (Invitrogen).

Quantitative PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems). The total reaction volume used for the analysis of *CtCAldOMT1* gene expression was of 50 μL, containing 1 μL cDNAs, 200 nM gene-specific primers and 25 μL 2 × SYBR Green Universal Master Mix (Applied Biosystems), with a preliminary step of 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 15 s, and of 60 ºC for 1 min. The PCR primers used were: forward primer, 5'-CTCAGCTACCAAAGGCAGACAA-3';
reverse primer,

5'-GCTAGCCAATAAGCGGCAGAT-3'. To check the specificity of annealing the oligonucleotides, dissociation kinetics was performed by the machine in the end of the experiment. For the expression of *CtCoAOMT1*, *3* and *5*, the reaction volume was of 50 μL containing 1 μL cDNAs, 900 nM gene-specific primers, 250 nM TaqMan probe, and 25 μl 2 × TaqMan Universal Master Mix (Applied Biosystems), with a preliminary step of 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The PCR primers were: *CtCoAOMT1*, forward primer, 5'-ATTCGGCACGAGGCTTCTC-3', reverse primer, 5'-GTGGCTGCCATTGGAATTG-3'; *CtCoAOMT3*, forward primer, 5'-GGCCTTCCTTCAACTTGAACAC-3', reverse primer, 5'-CCGCCATGTGATCGGTTT-3'; *CtCoAOMT5*, forward primer, 5'-GGCACGAGGAAAAACCAAGTAA-3', reverse primer, 5'-GTGCGTCACTCTGCAATAAGCT-3'. The TaqMan probes were: *CtCoAOMT1*, 5'-CCACCACAAATCTAT-3'; *CtCoAOMT3*, 5'-AGTAAACCTCAAGTAACCG-3'; *CtCoAOMT5*, 5'-CGACTCAAACAACGAAG-3'. Primers and probes were designed with a Primer Express Software (Applied Biosystems). For quantification, standard curves were prepared with each recombinant plasmid containing full-length *CtCAldOMT1* and *CtCoAOMT1*, *3* and *5* sequences. The 18S ribosomal RNA was analyzed as an internal standard, to normalize the transcript abundance in each sample.

Methanol extracts of C. tinctorius stems

Freeze-dried *C. tinctorius* stems were cut with scissors and then extracted with hot methanol (MeOH). The MeOH extract was first treated with β-glucosidase, then submitted to trimethylsilylation, followed by GC-MS analysis.

Expression of recombinant CtCoAOMTs and CtCAldOMT in E. coli

The coding sequences of *CtCoAOMT1*, *3* and *5*, and *CtCAldOMT1* were amplified by a polymerase chain reaction (PCR) with primers designed to introduce *Nde*I and *Not*I sites for *CtCoAOMT1* and *3*, and *CtCAldOMT1*, and *Bbs*I and *Not*I sites for *CtCoAOMT5*, immediately upstream their start and stop codons. The PCR products were first cloned into pCR2.1 vectors (Invitrogen). After confirming their sequence accuracy, the coding regions of the clones were subcloned into pET23 (Novagen) expression vectors. Each construct was transferred into *E. coli* BL21 (DE3) cells (Novagen). The induction and expression of recombinant CtCoAOMT1, 3 and 5, and CtCAldOMT1 were conducted according to Li *et al*.[15] After harvesting by centrifugation (2000×g for 10 min), the cell pellets were processed for affinity purification of CtCoAOMT1, 3 and 5, and CtCAldOMT1 proteins, with the His-Bind Resin affinity purification system (Novagen), according to the manufacturer's protocol.

Substrate specificity of recombinant CtCoAOMTs and CtCAldOMT

The basic reaction mixture contained 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 40 μM SAM, 0.1 mM substrate and an enzyme solution in a final volume of 200 μL. All reactions were performed at 30 ºC for 1 h. After incubation, the enzyme assay products were analyzed by GC-MS, as previously described.[11] To characterize the enzyme pH optimum values, the substrate and each recombinant enzyme were used in phosphate (pH 6.0–8.0) and Tris-HCl (pH 7.5–9.0) buffers. Substrate specificities of the recombinant CtCoAOMTs (CtCoAOMT1, 3 and 5) and CtCAldOMT (CtCAldOMT1) were investigated *in vitro*, with the following phenylpropanoid substrates: CA, 5-HFA, CaCoA, 5-HFCoA, CaAld, 5-HCAld, CaAlc and 5-HCAlc. The quantification of the reaction products was conducted by a stable-isotope dilution method, according to Sakakibara *et al*.[10]

Enzyme reaction kinetics of recombinant CtCoAOMTs and CtCAldOMT

To determine the V_{max} and apparent K_m values of CtCoAOMT1, 3 and 5, CaCoA (4.0–3000 μM) and 5-HFCoA (4.0–500 μM) were used as substrates in the CoAOMT reaction. For the values of CtCAldOMT1, CA (2.5–200 μM), 5-HFA (2.5–200 μM), CaCoA (4.0–1000 μM), 5-HFCoA (4.0–400 μM), CaAld (2.5–200 μM), 5-HCAld (2.5–200 μM), CaAlc (2.5–200 μM) and 5-HCAlc (2.5–200 μM) were used as substrates. All reactions were performed at 30 ºC for 20 min, each at optimum pH. All incubations contained 200 μM SAM as a methyl group donor. The V_{max} and K_m values were determined from Lineweaver-Burk plots.

For inhibiting the kinetics of CtCAldOMT1, 5-HCAld (0–20 μM) was used to assay the 5-HCAld-induced inhibition of CAldOMT-mediated methylation of 5-HFA,

CaAld, CaAlc and 5-HCAlc. On the other hand, 5-HFA (0–300 μM), CaAld (0–200 μM), CaAlc (0–300 μM) and 5-HCAlc (0–200 μM) were used to assay the inhibition of CAldOMT-mediated methylation of 5-HCAld. The reaction was carried out in 50 mM Tris-HCl, pH 8.0. The K_i and K_i' values were determined from replots of the Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Cloning of CtCoAOMT and CtCAldOMT cDNAs

Screening of the λZAP II cDNA libraries prepared from maturing seeds of *C. tinctorius* with *PtreCAldOMT* and *PtaeCoAOMT* cDNAs resulted in the isolation of six full-length *CtCoAOMT* cDNAs and one *CtCAldOMT* cDNA.

The open reading frame of *CtCAldOMT1* was of 1068 bp, encoding a 40 kD protein. The deduced amino acid sequence of CtCAldOMT1 was 80% identical to that of *Zinnia elegans* COMT and ca. 70% identical to those of other COMTs. Phylogenetic analysis of CtCAldOMT1 and available full-length plant OMT protein sequences showed that CtCAldOMT1 belongs to the CAldOMT (COMT) cluster (Fig. 2).

The open reading frames of the six *CtCoAOMT*s were 732–756 bp, encoding 28–29 kD proteins. The identity of these six deduced proteins ranged from 79 to 98% (Table 1). Phylogenetic analysis of CtCoAOMTs and available full-length OMT protein sequences showed that all CtCoAOMTs belong to the CoAOMT cluster (Fig. 2).

Table 1
Overall relationships (% identity) of the six CtCoAOMTs

	CtCoAOMT1	CtCoAOMT2	CtCoAOMT3	CtCoAOMT4	CtCoAOMT5	CtCoAOMT6
CtCoAOMT1		98	94	93	81	84
CtCoAOMT2			94	93	80	82
CtCoAOMT3				96	79	82
CtCoAOMT4					79	80
CtCoAOMT5						91
CtCoAOMT6						

CoAOMTs belong to plant class I OMT[16] (= Type II OMT[17]). Recently, Ibdah *et al*.[18] cloned a novel plant class I OMT from *Mesembryanthemum crystallinum* (ice plant), designated as PFOMT. The amino acid sequences of CtCoAOMTs and PFOMT were compared, showing no high sequence identity, *e.g*. the sequence identity of CtCoAOMT5 to PFOMT was of 54% (Fig. 2).

CtCoAOMTs were divided into three subclasses (Fig 2). The most closely related OMT from other plants for CtCoAOMT1 was *Z. elegans* CoAOMT, at a 94% score. In addition, CtCoAOMT3 was also related to *Z. elegans* CoAOMT at a 94% score. On the other hand, CtCoAOMT5 was only 79% identical to *Z. elegans* CoAOMT and 86% identical to *Codonopsis lanceolata* CoAOMT.

Among the six CtCoAOMTs, one from each subclass in the phylogenetic tree (CtCoAOMT1, CtCoAOMT3 and CtCoAOMT5) was further characterized, as the number of the corresponding cDNAs isolated in the screening was larger than that of the other members of each subclass.

Expression analysis of CtCoAOMTs and CtCAldOMT in maturing seeds and stems of C. tinctorius

In a subsequent step, the expression of the mRNAs encoding CtCoAOMT1, 3 and 5, and CtCAldOMT1 was examined with a quantitative real-time PCR and total RNA isolated from seeds – in various maturation stages (3 to 18 DAF) – and stems. As shown in Figure 3, the *CtCoAOMT3* expression was much higher than those of *CtCoAOMT1* and *CtCoAOMT5*, which were almost negligible [*CtCoAOMT1*, 0.002742×10^6 copies/μg of RNA at 9 DAF and *CtCoAOMT5*, 0.000286×10^6copies/μg of RNA at 12 DAF]. Their expression increased from 3 to 9 DAF, after which they decreased. Similarly, the *CtCAldOMT1* expression increased rapidly from 3 to 6 DAF and decreased after 9 DAF. The amount of *CtCAldOMT1* transcript at 9

DAF was ca. 5 times higher than that of *CtCoAOMT3*. Importantly, the time course of the expression matches[10] the pattern of lignin deposition in the seeds (Fig. 3).

Furthermore, the expressions of *CtCoAOMT3* and *CtCAldOMT1* were significant in the stem, where lignins, and not lignans, are biosynthesized, while the expressions of *CtCoAOMT1* and *CtCoAOMT5* were almost negligible (Table 2). On the other hand, matairesinol and arctigenin, occurring as major lignans in the seeds,[10] were not detected in the MeOH extracts of *C. tinctorius* stems after the β-glucosidase treatment (data not shown). Taken together, these results strongly suggest that *CtCoAOMT3* and *CtCAldOMT1* are involved at least in lignin biosynthesis.

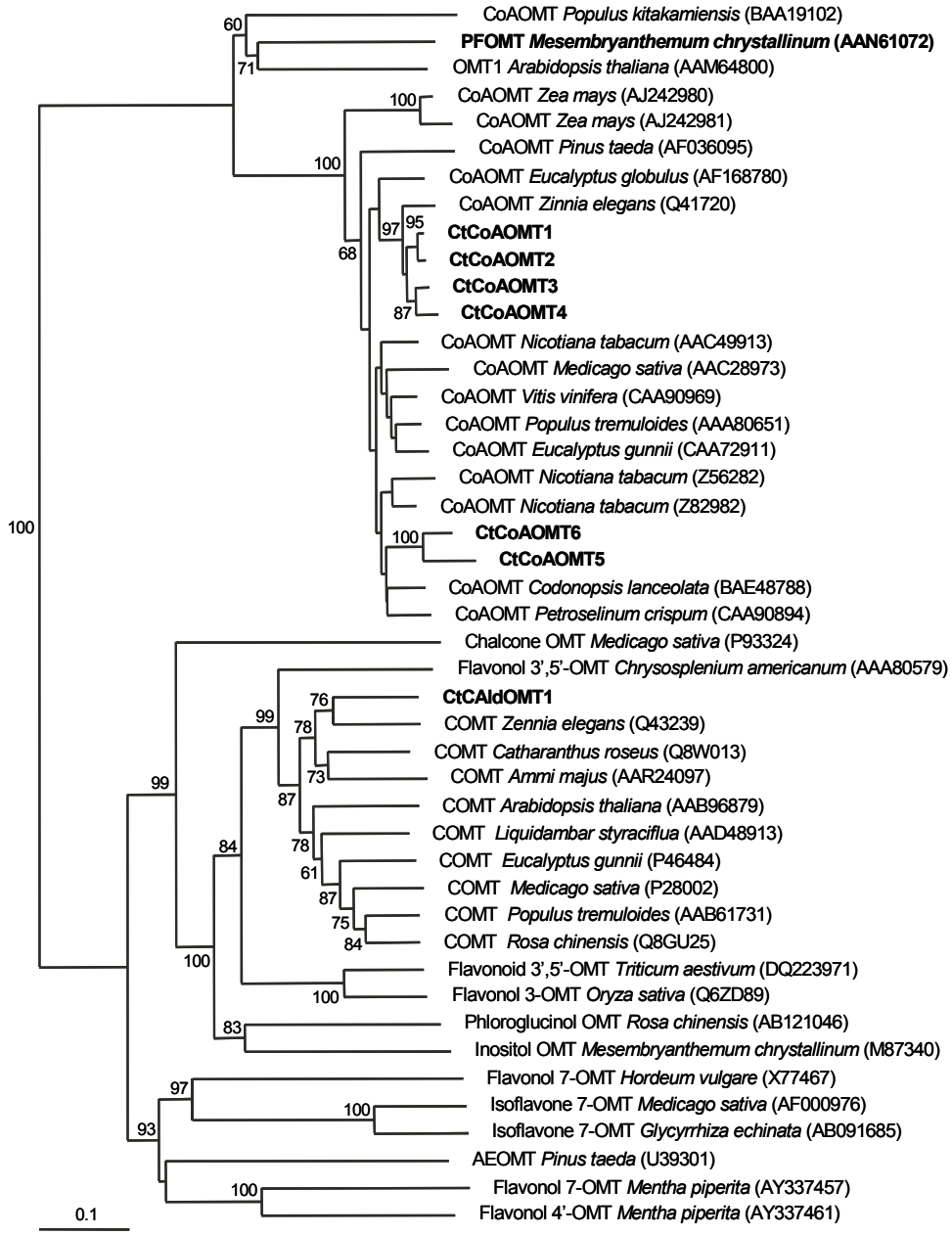

Figure 2: Relationship tree of the plant OMTs selected. The numbers at forks are bootstrap values indicating the percent values necessary for obtaining this particular branching, in 1 000 repetitions of the analysis. Only values exceeding 60% are shown

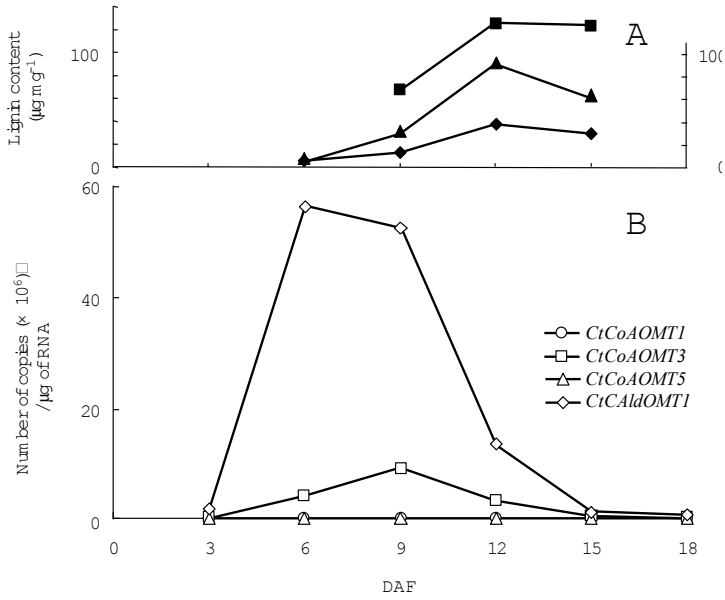

Figure 3: Gene expressions of *CtCoAOMT*s and *CtCAldOMT*, lignin contents and nitrobenzene oxidation products in maturing seeds of *C. tinctorius*: A – amounts of lignin and nitrobenzene oxidation products; ▲ – vanillin; ♦ – syringaldehyde; ■ – lignin content measured by the acetyl bromide method;[10] B – gene expression of *CtCoAOMT*s and *CtCAldOMT*. Transcript levels, analyzed by RT-PCR, are given as absolute values, after being normalized to the 18S ribosomal RNA levels. Copy number of each isoform was determined with the recombinant plasmid containing the corresponding full-length sequences. The values represent the averages of duplicate experiments

Table 2
Gene expression of *CtCoAOMT*s and *CtCAldOMT* in *C. tinctorius* stems

	Number of copies ($\times 10^7$)/μg of RNA
CtCoAOMT1	0.0000561
CtCoAOMT3	9.36
CtCoAOMT5	0.00162
CtCAldOMT1	9.32

Kinetic properties and roles of CtCoAOMTs and CtCAldOMT1

To characterize the biochemical reactions catalyzed by CtCoAOMTs and CtCAldOMT1, their recombinant proteins in *E. coli* cells were expressed. The purified recombinant CtCoAOMT1, 3 and 5, and CtCAldOMT1 were tested *in vitro* for their activity towards 8 monomeric phenyl-propanoid substrates: CA, 5-HFA, CaCoA, 5-HFCoA, CaAld, 5-HCAld, CaAlc and 5-HCAlc. GC-MS analysis of the reaction products showed that CtCoAOMT1, 3 and 5 catalyze the *O*-methylation of the 3- and 5-hydroxyl groups of CaCoA and 5-HFCoA, respectively. However, when other substrates were employed, no significant activities were detected. Therefore, it was confirmed that CtCoAOMT1, 3 and 5 were CoAOMTs that catalyze the *O*-methylation of CaCoA and 5-HFCoA.

Next, the kinetic analysis of these three CtCoAOMTs was conducted by a GC-MS-based assay system, with CaCoA and 5-HFCoA as substrates. As summarized in Table 3, the k_{cat}/K_m values for CaCoA and 5-HFCoA of CtCoAOMT5 were extremely low, as compared to those of CtCoAOMT1 and CtCoAOMT3.

Table 3
Kinetic properties of purified recombinant CtCoAOMTs and CtCAldOMT

	Substrate	K_m (μM)	K_{cat}/K_m (μM^{-1}m in $^{-1}$)
CtCoAOMT1	CaCoA	8.1	0.019
	5-HFCoA	12.2	0.016
CtCoAOMT3	CaCoA	63.3	0.097
	5-HFCoA	6.8	0.157
CtCoAOMT5	CaCoA	994.8	0.00003
	5-HFCoA	223.3	0.00023
CtCAldOMT1	CA	103.3	0.040
	5-HFA	63.0	0.385
	CaCoA	555.6	0.004
	5-HFCoA	374.5	0.004
	CaAld	51.9	0.261
	5-HCAld	20.2	0.378
	CaAlc	17.7	0.379
	5-HCAlc	48.4	0.313

Abbreviations of compounds are those used in Fig. 1

As McPFOMT, classified as a plant class I OMT like CoAOMTs, can methylate both flavonoids and CaCoA,[18] the CtCoAOMTs was tested as to its capacity of catalyzing the methylation of flavonoids. However, CtCoAOMT3 and CtCoAOMT5 did not show methylation activity towards apigenin, a major flavonoid compound in *C. tinctorius* seeds. CtCoAOMT1 was not subjected to the flavonoid assay.

Taken together, the present results indicate that CtCoAOMT3 is involved in lignin biosynthesis during stem and seed development, by methylating CaCoA, which gives feruloyl CoA. On the other hand, the roles of CtCoAOMT1 and CTCoAOMT5 remain unclear. CtCoAOMT1 might be involved in defense responses, but it is unlikely that CtCoAOMT5 has such a role, once its kinetic constants towards CaCoA and 5-HCoA are very poor and because it did not catalyze the methylation of CA, 5-HFA, CaAld, 5-HCAld, CaAlc and 5-HCAlc.

CAldOMTs belong to the plant class II OMT[16] (= Type I OMT[17]), which generally shows wide substrate specificities.[19] The present study showed that CtCAldOMT1 catalyzes the *O*-methylation of all possible substrates on the cinnamate/monolignol pathway (CA, 5-HFA, CaCoA, 5-HFCoA, CaAld, 5-HCAld, CaAlc and 5-HCAlc), which agrees well with the general character of class II OMTs, *i.e.* a wide substrate specificity. The kinetic constants of CtCAldOMT1 shown in Table 3 clearly indicate that acids, aldehydes and alcohols are good substrates, showing comparable k_{cat}/K_m values, except for CA. Among these substrates, 5-HFA, CaAlc and 5-HCAld exhibited larger k_{cat}/K_m values (5-HFA, 0.385; CaAlc, 0.379; and 5-HCAld, 0.378 $\mu M^{-1}\cdot min^{-1}$), while CaAlc and 5-HCAld showed smaller K_m values (CaAlc, 17.7; 5-HCAld, 20.2 μM). On the other hand, the CoA esters, CaCoA and 5-HFCoA with large K_m and small k_{cat}/K_m values are poor substrates, which is a general characteristic of CAldOMTs.

Next, an inhibition kinetic analysis was developed to determine the most suitable substrate, once Li *et al.* reported[15] that *O*-methylation of CA and 5-HFA by CAldOMTs of several angiosperm tree species was inhibited effectively by 5-HCAld. For example, they showed[15] that PtreCAldOMT-catalyzed methylations of 5-HFA and CA were intensely inhibited by 5-HCAld (competitive inhibition, K_i for 5-HFA, 0.26μM, and for CA, 2.1μM), while the methylation of 5-HCAld was only weakly inhibited by acids (competitive inhibition, K_i for 5-HFA 110.3 μM, and for CA 121.7 μM). Although the present study demonstrates the high affinity of CtCAldOMT1 towards the five substrates

(5-HFA, CaAld, 5-HCAld, CaAlc and 5-HCAlc) of the cinnamate/monolignol pathway (Table 3), no inhibition kinetic analyses of CAldOMT made with all these substrates were reported.[11,15] Therefore, the five compounds were used as substrates for the inhibition kinetic assays. As summarized in Tables 4 and 5, 5-HCAld strongly inhibited the CtCAldOMT1-catalyzed methylation of the other compounds (Table 4). In contrast, methylation of 5-HCAld was not inhibited by 5-HFA, being only weakly inhibited by CaAld, CaAlc and 5-HCAlc (Table 5). All these results, obtained by the utilization of all possible substrates of the cinnamate/monolignol pathway, indicated that 5-HCAld was the best substrate for CAldOMT, which agrees well with the previous results[15] obtained with CA, 5-HFA and 5-HCAld.

Table 4
Inhibition kinetic analysis of CtCAldOMT1.
Kinetic parameters of inhibition for the methylation of each substrate by 5-HCAld

Substrate	K_i(μM)	K_i' (μM)	Type of inhibition
5-HFA	1.5	3.2	mixed
CaAld	32.3	2.8	uncompetitive
CaAlc	15.6	1.0	uncompetitive
5-HCAlc	2.0	13.0	mixed

Table 5
Inhibition kinetic analysis of CtCAldOMT1.
Kinetic parameters of the inhibition of 5-HCAld methylation by each substance

Inhibitor	K_i(μM)	K_i' (μM)	Type of inhibition
5-HFA	-	-	-
CaAld	93.4	66.7	mixed
CaAlc	116.8	817.2	mixed
5-HCAlc	461.9	197.6	mixed

Abbreviations of compounds are those used in Fig. 1

Methylation of 5-HCAld by CAldOMTs is the key step towards syringyl lignin biosynthesis.[15] Our recent results indicated[11] that the best substrate for *Arabidopsis thaliana* CAldOMT could be 5-HCAld, as well, indicating the involvement of the OMT in syringyl lignin biosynthesis.[11] In addition, such results strongly suggested[11] that AtCAldOMT is also involved in the *O*-methylation of the C3 position of 3,4-dihydroxyphenyl of CaAld and/or CaAlc in *A. thaliana*. Furthermore, significant amounts of CaAlc were detected in *A. thaliana*. On the other hand, the present kinetic data showed that 5-HCAld inhibited strongly the CtCAldOMT1-catalyzed methylation of 3,4-dihydroxyphenyl compounds (uncompetitive inhibition, against CaAld, K_i 32.3 μM and K_i' 2.8 μM; against CaAlc, K_i 15.6 μM and K_i' 1.0 μM), while CaAld and CaAlc were not good inhibitors for 5-HCAld methylation (mixed type inhibition, CaAld, K_i 93.4 μM and K_i' 66.7 μM; CaAlc, K_i 116.8 μM and K_i' 817.2 μM). These results suggested that CaAld and CaAlc are not good *in vivo* substrates, which agrees with the absence of CaAld and CaAlc in maturing seeds of *C. tinctorius*.[10] In addition, the results suggested that the roles of CaAld and CaAlc in the lignin biosynthesis might be different in different plant species, although further experiments are still necessary.

Besides guaiacyl lignin, lignans are biosynthesized from ConAlc. The biosynthetic pathway for ConAlc, dedicated to lignan biosynthesis, and the genes involved remain unclear.[1,20] Recently, based on a series of stable-isotope tracer experiments, Sakakibara *et al.*[10] suggested that lignan biosynthesis in *C. tinctorius* proceeded *via* FA, and that FA is produced by *O*-methylation of CA. As CtCAldOMT1 exhibits a moderate activity towards CA, the

OMT might be involved in lignan biosynthesis in maturing seeds of *C. tinctorius*. The observation might be further supported[10] by the fact that lignan biosynthesis in the seeds starts at 7 DAF, just after the rapid increase of *CtCAldOMT1* expression. However, the presence of a novel lignan-specific OMT in the cinnamate/monolignol pathway is also possible. In fact, a novel class II OMT was isolated by screening the *C. tinctorius* cDNA library under low-stringency conditions, with *CtCAldOMT1* as a probe (T. Nakatsubo *et al*., manuscript in preparation). Thus, identification of the *C. tinctorius* OMT responsible for the formation of FA for lignan biosynthesis awaits further studies.

CONCLUSIONS

The function of three *CtCoAOMT* genes (*CtCoAOMT1, 3 and 5*) and that of a *CtCAldOMT* gene (*CtCAldOMT1*) were investigated. Based on enzyme kinetic and gene expression analyses, *CtCoAOMT3* and *CtCAldOMT1* were found to be involved in lignin biosynthesis.

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