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Short Communication

A new *O*-methyltransferase for monolignol synthesis in *Carthamus tinctorius*

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Abstract A novel type of *O*-methyltransferase (OMT) cDNA was isolated from maturing seeds of *Carthamus tinctorius* (safflower). The deduced sequence of the OMT protein showed moderate sequence identity (52%) with *C. tinctorius* 5-hydroxyconiferaldehyde *O*-methyltransferase 1 (CAldOMT1). Phylogenetic analysis showed that the novel OMT did not belong to the typical CAldOMT [=caffeic acid OMT (CAOMT)] cluster. The recombinant protein of the OMT catalyzed 3- (or 5-) *O*-methylation of hydroxycinnamaldehydes and hydroxycinnamyl alcohols, while it showed only weak or moderate activity toward hydroxycinnamates and hydroxycinnamoyl coenzyme A esters. Therefore, this OMT was designated as *C. tinctorius* 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol OMT (CtAAOMT). The time profile of *CtAAOMT* gene expression in *C. tinctorius* matched the patterns of lignin accumulation. Taken together, our data strongly suggest that along with CtCAldOMT1, CtAAOMT is involved in biosynthesis of syringyl lignin.

Key words: *O*-Methyltransferase, *Carthamus tinctorius*, lignin, monolignol, flavonoid.

Plant *O*-methyltransferases (OMTs) are involved in the biosynthesis of various plant secondary metabolites including lignins, lignans, and flavonoids (Ragamustari et al. 2013, Ragamustari et al. 2014; Schröder et al. 2002; Umezawa 2003; Umezawa 2010; Umezawa et al. 2013) and expand the structural diversity of natural products. Three OMTs have so far been identified as key enzymes involved in monolignol biosynthesis (Figure 1): caffeoyl CoA *O*-methyltransferase (CCoAOMT) (Zhong et al. 1998; Zhong et al. 2000), caffeic acid *O*-methyltransferase (CAOMT=CAldOMT) (Bugos et al. 1991; Dwivedi et al. 1994; Li et al. 2000; Zhong et al. 1998), and hydroxycinnamic acids/hydroxycinnamoyl CoA esters OMT (AEOMT) (Li et al. 1997; Moyle et al. 2002). Up to now, CAldOMTs have been found in angiosperms, while CCoAOMTs have been reported from both gymnosperms and angiosperms (Umezawa 2010). On the other hand, AEOMTs have so far been reported only from pine trees (Li et al. 1997; Moyle et al. 2002; Umezawa 2010). Initially, the role of CAOMTs in the cinnamate/monolignol pathway which gives monolignols from phenylalanine (Figure 1) had been assigned to methylation of caffeic and 5-hydroxyferulic acids to the ferulic and sinapic acids, respectively (Umezawa 2010). Later, the true function of CAOMT was found to be methylation of 5-hydroxyconiferaldehyde to give sinapaldehyde, and it was renamed as 5-hydroxyconiferaldehyde OMT (CAldOMT) (Li et al. 2000, Li et al. 2001; Osakabe et al. 1999; Umezawa 2010). The CAldOMT-catalyzed methylation of caffealdehyde to give coniferaldehyde has also been reported in *Medicago sativa* (Guo et al. 2001; Parvathi et al. 2001), as well as being implicated in *Arabidopsis thaliana* (Nakatsubo et al. 2008).

Maturing seeds of *Carthamus tinctorius* (safflower) accumulate several phenylpropanoid secondary metabolites including lignins, lignans, and flavonoids (Bae et al. 2002; Nakatsubo et al. 2007; Sakakibara et al. 2007; Sakamura et al. 1980; Umezawa et al. 2013), and

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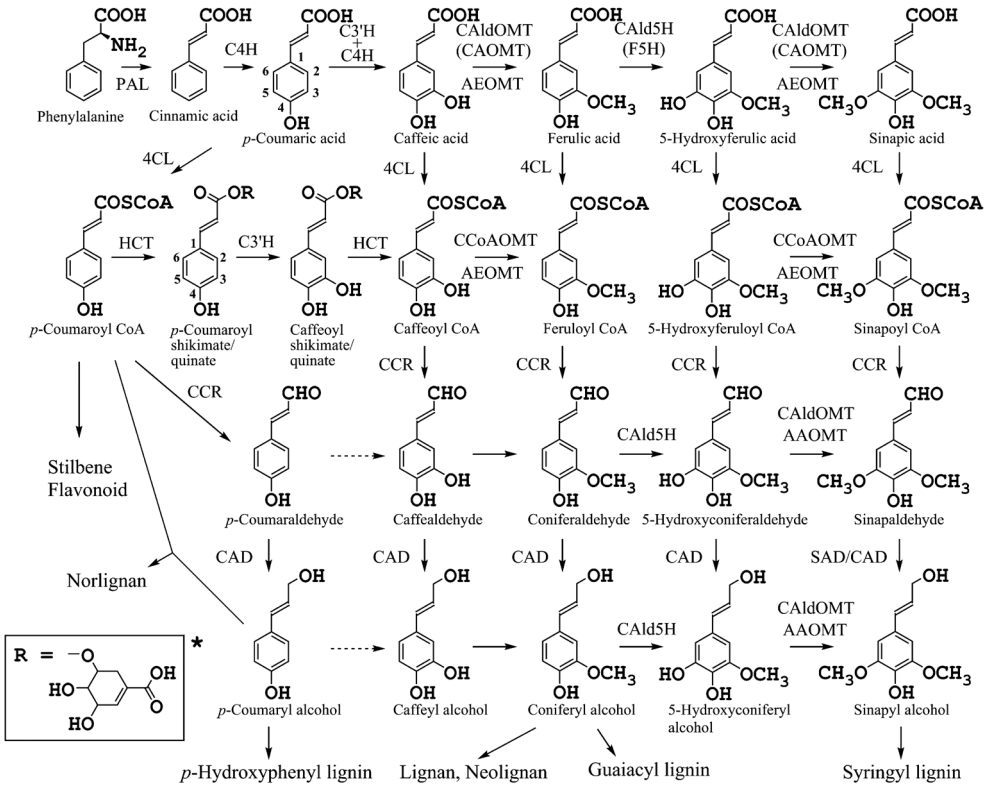

Figure 1. The cinnamate/monolignol pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3′H, *p*-coumaroyl shikimate/quinate 3-hydroxylase; CAldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; CAOMT, caffeic acid *O*-methyltransferase; AEOMT, hydroxycinnamic acids/hydroxycinnamoyl CoA esters *O*-methyltransferase; CAld5H, coniferaldehyde 5-hydroxylase; F5H, ferulate 5-hydroxylase; 4CL, 4-hydroxycinnamate CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; AAOMT, 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol *O*-methyltransferase. *: Only the structure of shikimate ester is shown.

their biosyntheses are initiated at particular points of seed maturation (Nakatsubo et al. 2007; Sakakibara et al. 2007; Umezawa et al. 2013). In addition, several protocols for *Agrobacterium*-mediated genetic transformation of the plant have been reported (Belide et al. 2011; Motamedi et al. 2011; Nykiforuk et al. 2012; Shilpa et al. 2010). This species can therefore be used for comparative studies of lignin and lignan biosynthesis. Previously, we characterized OMTs that were present in maturing seeds of *C. tinctorius* and we identified the cDNAs of OMTs most probably involved in lignin biosynthesis: *CtCCoAOMT3* (*CtCoAOMT3*) and *CtCAldOMT1* (Nakatsubo et al. 2007), lignan methylation: *C. tinctorius matairesinol OMT* (*CtMROMT*) (Umezawa et al. 2013), and flavonoid methylation: *C. tinctorius flavonoid OMT* (*CtFOMT*) and *CtMROMT* (Umezawa et al. 2013). Herein, we report the biochemical characterization of a new *C. tinctorius* OMT that shares moderate amino acid sequence identity (ca. 50%) with typical CAldOMTs (CAOMTs). The OMT showed substrate preferences toward 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, and therefore was named *C. tinctorius* 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol OMT (CtAAOMT).

A λZAP II cDNA library made from *C. tinctorius* cv. Round-leaved White seeds (Nakatsubo et al. 2007; Umezawa et al. 2013) was screened using *CtCAldOMT1* cDNA (GenBank AB430466) (Nakatsubo et al. 2007) as a probe in the same manner as reported previously (Nakatsubo et al. 2007; Umezawa et al. 2013). Seven positive cDNA fragments were obtained after two rounds of plaque purification. End-sequencing of the positives revealed that six clones encoded CtCAldOMT1, while the other clone was not identical to *CtCAldOMT1*, named *CtAAOMT* following its biochemical characterization described below. The cDNA (DDBJ accession number, AB980250) encodes a polypeptide of 367 amino acids, which is shown in Figure 2 together with other selected OMTs including CtCAldOMT1, CtMROMT, CtFOMT, *Forsythia koreana* MROMT (FkMROMT) (Ragamustari et al. 2014), and *M. sativa* isoflavone OMT. The *S*-adenosyl-L-methionine (SAM)-binding motifs A, B, and C (Joshi and Chiang 1998); and domains I, II, III, IV, and V that are conserved among plant OMTs (Dunlevy et al. 2010) were present in CtAAOMT (Figure 2). Phylogenetic analysis, which was conducted as previously (Ragamustari et al. 2013; Umezawa et al. 2013) (Figure 3) indicated that the CtAAOMT showed 55% identity to FkMROMT, while it exhibited 49–53% identity to

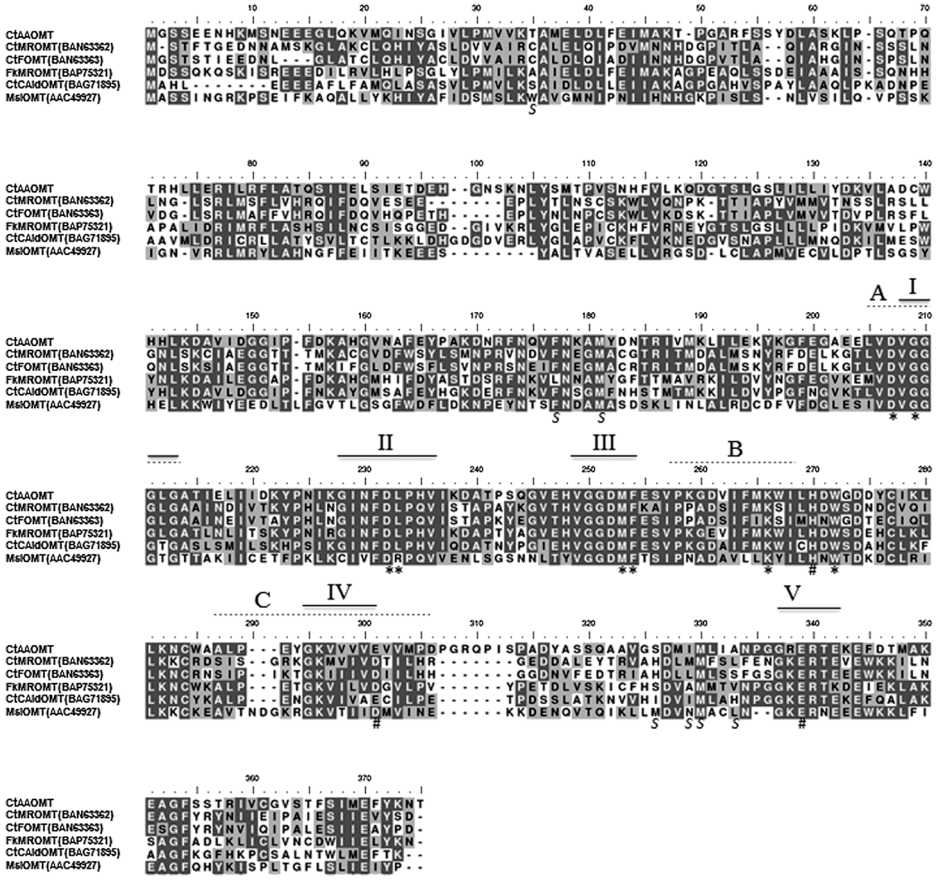

Figure 2. Alignment of the predicted amino acid sequences of CtAAOMT, CtCAldOMT1, CtMROMT, CtFOMT and related OMTs. SAM-binding motifs A, B, and C (Joshi and Chiang 1998); and regions I, II, III, IV, and V are domains conserved among plant OMTs (Dunlevy et al. 2010). #, Catalytic residues; *, SAM binding residues; S, Substrate binding residues of *Medicago sativa* isoflavone OMT (MsIOMT, AAC49927) as determined by Zubieta et al. (2001).

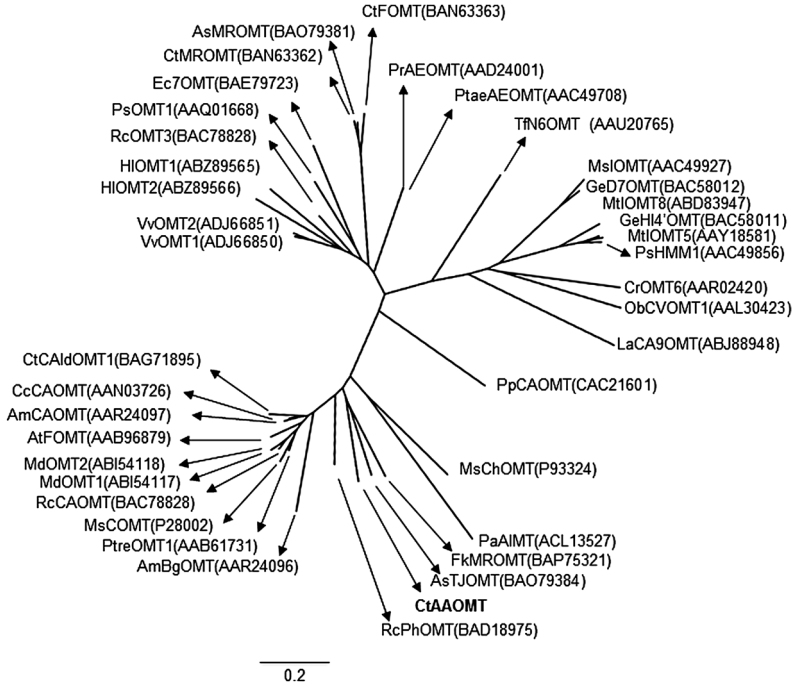

Figure 3. Phylogenetic analysis of CtAAOMT and related OMT proteins. GenBank accession numbers for the proteins are shown in parentheses beside each name.

CAOMTs (CAldOMTs) from various plant species, including CtCAldOMT1 (52%) (Nakatsubo et al. 2007). The phylogenetic relationships (Figure 3) indicated that CtAAOMT did not belong to the CAldOMT (CAOMT) group, indicating that CtAAOMT is a novel type of plant OMT.

C. tinctorius cv. Round-leaved White was cultivated and the seeds, stems, and leaves were subjected to determination of the abundance of transcripts using quantitative real-time polymerase chain reaction (qRT-PCR) with the 18S ribosomal RNA as internal standard to normalize the transcript abundance in each sample as previously (Ragamustari et al. 2013; Umezawa et al. 2013) but with specific primers for *CtAAOMT*. Figure 4A shows that the *CtAAOMT* transcript abundance in seeds increased from 3 days after flowering (DAF) to 6 DAF and thereafter decreased. The profile is in good accordance with lignin accumulation in *C. tinctorius* seeds, which starts at 6 DAF and then levels off (Sakakibara et al. 2007). Therefore, the *CtAAOMT* gene expression pattern in seeds was synchronized with the profile of lignin accumulation. In addition, the *CtAAOMT* gene was significantly expressed in stem where lignins are synthesized, which is in contrast with the expression of *CtFOMT* (Figure 4A) and *CtMROMT* (Umezawa et al. 2013).

Next, a recombinant protein of *CtAAOMT* was

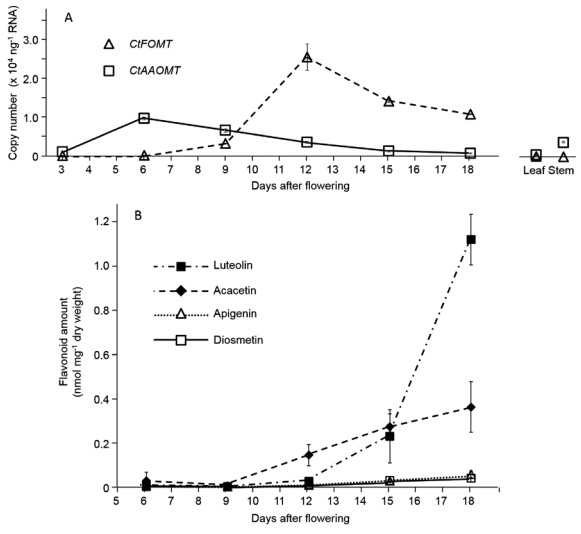

Figure 4. Time course of *CtFOMT* and *CtAAOMT* expression and flavonoid accumulation in *C. tinctorius*. A: Expression of *CtAAOMT* in *C. tinctorius* maturing seeds, stem, and leaf. Transcript levels are shown as absolute values after being normalized to 18S ribosomal RNA levels. Copy numbers of *CtAAOMT* were determined using a standard curve constructed using the recombinant plasmid containing the corresponding full-length sequence. B: Flavonoid accumulation in maturing seeds of *C. tinctorius*. *CtFOMT* expression and accumulation of apigenin and acacetin (Umezawa et al. 2013) are shown for comparison. Error bar represents standard deviation [number of replicates (n)=3]. The values of diosmetin might be incorporated slightly with those of chrysoeriol due to poor HPLC separation.

prepared and subjected to biochemical characterization as previously reported (Nakatsubo et al. 2007; Umezawa et al. 2013). Briefly, the coding sequence of *CtAAOMT* was amplified by PCR using primers designed to introduce *Nde*I and *Not*I sites immediately upstream of the start and stop codons. The PCR products were first cloned into pCR2.1 vectors (Invitrogen Life Technologies Corp., Carlsbad, CA, USA). After confirming sequence accuracy, coding regions of the vectors were subcloned into pET23a (Novagen, San Diego, CA, USA) expression vectors with their restriction sites. Each construct was transferred into *Escherichia coli* BL21 (DE3) strain (Novagen). The induction and expression of recombinant CtAAOMT and its purification using the His·Bind Resin affinity purification system (Novagen) were conducted as previously (Nakatsubo et al. 2007; Umezawa et al. 2013). The purified recombinant CtAAOMT was tested in vitro for its activity toward eight phenylpropanoid monomers: caffeic acid, 5-hydroxyferulic acid, caffeoyl CoA, 5-hydroxyferuloyl CoA, caffealdehyde, 5-hydroxyconiferaldehyde, caffeyl alcohol, and 5-hydroxyconiferyl alcohol. Preparation of the phenylpropanoid monomers was reported previously (Nakatsubo et al. 2007; Nakatsubo et al. 2008). The reaction mixture (total volume 200 μl) consisted of 50 mM potassium phosphate (pH 7.0), 2 mM $MgCl_2$, 40 μM SAM, 0.1 mM substrate, and the appropriate volume of enzyme solution. After incubating at 30°C for 30 min, the reaction products were extracted with ethyl acetate. In separate experiments, the same assay was conducted without the 2 mM $MgCl_2$. The ethyl acetate extracts were dried and subjected to gas chromatography-mass spectrometry (GC-MS), which was conducted with a Shimadzu QP-5050A GC-MS system (Shimadzu Co., Kyoto, Japan) as previously (Nakatsubo et al. 2007). The incubation of each of the eight phenylpropanoid monomers with CtAAOMT gave the corresponding 3- or 5-mono-*O*-methylated products, which were identified by GC-MS analysis by comparing their retention times and mass spectra with those of authentic samples. Addition of Mg^{2+} ion into the assay mixture did not enhance the activity.

The V_{max} and K_m values of CtAAOMT were determined exactly as previously (Nakatsubo et al. 2007) for the following substrates but with the concentration range and optimal pH shown in parentheses: caffeic acid (6.25–400 μM; 50 mM potassium phosphate buffer, pH 6.0), 5-hydroxyferulic acid (5.0–200 μM; 50 mM potassium phosphate buffer, pH 6.0), caffeoyl CoA (6.25–400 μM; 50 mM Tris/HCl, pH 7.5), 5-hydroxyferuloyl CoA (6.25–400 μM; 50 mM potassium phosphate buffer, pH 7.5), caffealdehyde (2.5–200 μM; 50 mM potassium phosphate buffer, pH 8.0), 5-hydroxyconiferaldehyde (2.5–200 μM; 50 mM potassium phosphate buffer, pH 7.5), caffeyl alcohol (2.5–200 μM; 50 mM potassium phosphate buffer, pH 8.0), and 5-hydroxyconiferyl

Table 1. Kinetic properties of purified recombinant CtAAOMT.

Substrate	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
Caffeic acid	2.3×10^2	2.0×10^{-3}
5-Hydroxyferulic acid	6.8×10	1.4×10^{-2}
Caffeoyl CoA	3.3×10^2	3.0×10^{-3}
5-Hydroxyferuloyl CoA	4.7×10	1.3×10^{-2}
Caffealdehyde	5.1×10	3.0×10^{-2}
5-Hydroxyconiferaldehyde	5.3×10	5.5×10^{-2}
Caffeyl alcohol	3.7×10	2.4×10^{-2}
5-Hydroxyconiferyl alcohol	1.9×10	9.9×10^{-2}

alcohol (2.5–200 μM; 50 mM potassium phosphate buffer, pH 7.5). All incubations contained 200 μM SAM as a methyl group donor. V_{max} and K_m values were determined from Lineweaver–Burk plots.

As expected from the moderate sequence homology of CtAAOMT with CAldOMTs, CtAAOMT showed a different substrate preference compared with typical CAldOMTs. As shown in Table 1, CtAAOMT showed the highest affinity (lowest K_m value) for 5-hydroxyconiferyl alcohol among the substrates used in this study. In addition, the k_{cat}/K_m value of CtAAOMT for 5-hydroxyconiferyl alcohol was the highest among the eight substrates, followed by 5-hydroxyconiferaldehyde (Table 1). On the other hand, when an equimolar mixture of 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol was incubated with CtAAOMT, the specific activity of 5-hydroxyconiferyl alcohol methylation by CtAAOMT was greatly reduced by 5-hydroxyconiferaldehyde; the activity was only 24% compared with the single substrate assay without the aldehyde, while the methylation activity of 5-hydroxyconiferaldehyde was inhibited slightly by 5-hydroxyconiferyl alcohol showing 78% activity compared with the single substrate assay without the alcohol. The results suggested that 5-hydroxyconiferaldehyde is a better substrate than 5-hydroxyconiferyl alcohol when both are present together. Thus, CtAAOMT had substrate preferences toward 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, whereas caffeic and 5-hydroxyferulic acids were poorer substrates. This is in sharp contrast to the substrate specificity of typical CAldOMTs that show high activities toward 5-hydroxyferulic acid as well as 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol (Koshiba et al. 2013; Li et al. 2000; Nakatsubo et al. 2007), although some grass CAldOMTs (CAOMTs) show varying substrate preferences (Koshiba et al. 2013). Moreover, CtAAOMT showed only the moderate sequence homology with CAldOMTs. Taken all together, the OMT was designated as 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol OMT (CtAAOMT).

The high expression of *CtAAOMT* in *C. tinctorius* seeds around 6–9 DAF (Figure 4A) was similar to those observed for *CtCAldOMT1* and *CtCCoAOMT3* (*CtCoAOMT3*) (Nakatsubo et al. 2007), which was in accordance with lignin accumulation around 9 DAF (Nakatsubo et al. 2007; Sakakibara et al. 2007). In addition, the *CtAAOMT* was expressed significantly in the stem where lignins are synthesized. Furthermore, the CtAAOMT-catalyzed 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol methylation gives rise to the essential compounds for syringyl lignin biosynthesis, sinapaldehyde/sinapyl alcohol (Figure 1), suggesting the role of CtAAOMT in syringyl lignin biosynthesis. Previously, we reported that CtCAldOMT1 is involved in the methylation of 5-hydroxyconiferaldehyde/5-hydroxyconiferyl alcohol and thereby syringyl lignin biosynthesis in *C. tinctorius* (Nakatsubo et al. 2007). The gene expression of *CtAAOMT* (9.6×10^3 ng^{-1} RNA, Figure 4) in seeds of *Carthamus* is only about 17% of *CtCAldOMT1* (5.6×10^4 ng^{-1} RNA, Nakatsubo et al. 2007) and the specificity constant (k_{cat}/K_m) of CtAAOMT toward 5-hydroxyconiferaldehyde is about 15% of CtCAldOMT1 (3.78×10^{-1} μM^{-1} min^{-1}, Nakatsubo et al. 2007). K_m value of CtAAOMT for 5-hydroxyconiferaldehyde was 5.3×10 μM, while that of CtCAldOMT1 for the same substrate was 2.0×10 μM. Taken together, we propose that CtCAldOMT1 plays a major role in the 5-*O*-methylation towards syringyl lignin biosynthesis in *C. tinctorius* and that CtAAOMT assists the function of CtCAldOMT1.

Because CAldOMTs (CAOMTs) often show significant methylation activity toward flavonoid compounds in addition to phenylpropanoid monomers (Lin et al. 2006; Muzac et al. 2000; Nakatsubo et al. 2008; Schröder et al. 2002; Umezawa 2010) and because CtMROMT efficiently methylates apigenin (flavonoid) in addition to the lignan matairesinol (Umezawa et al. 2013), it was of interest to know whether CtAAOMT shows flavonoid and lignan methylation activity (Figure 5). Previously, we detected several lignans and two flavonoids apigenin and acacetin in *C. tinctorius* seeds (Sakakibara et al. 2007; Umezawa et al. 2013), while it was reported that *C. tinctorius* seeds contained another flavonoid luteolin (Bae et al. 2002). Hence, we subjected two flavonoid compounds [apigenin and luteolin, purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)] and a lignan [matairesinol, prepared previously (Umezawa et al. 1992)] to CtAAOMT methylation assay as previously (Umezawa et al. 2013). The reaction products obtained following incubation of apigenin with CtAAOMT was submitted to liquid chromatography-mass spectrometry (LC-MS) analysis, which was conducted with a system consisted of a Shimadzu LC-10AD*vp* HPLC series liquid chromatograph and a Shimadzu LC/MS-2010A single quadrupole mass spectrometer (Shimadzu Co.), using a Supelcosil ABZ+Plus column (25 cm×2.1 mm, 5 μm, Supelco, Bellefonte, PA, USA) with a Supelguard ABZ+Plus guard column (2 cm×2.1 mm, Supelco). The

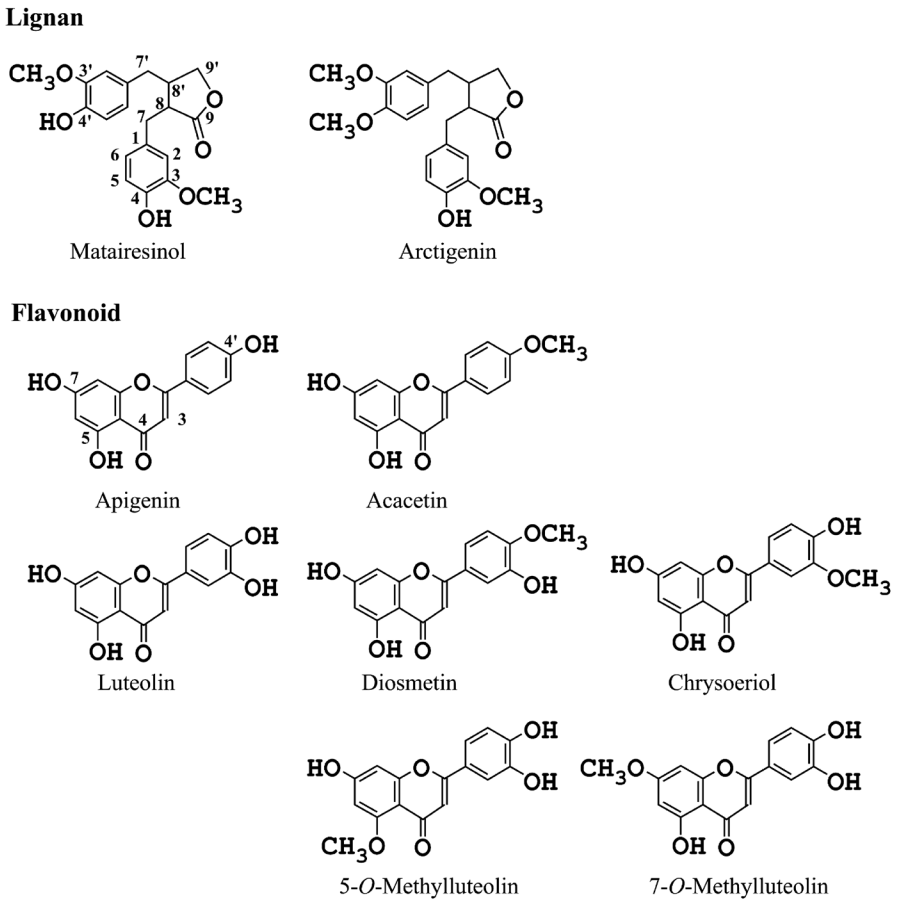

Figure 5. Structures of the compounds assayed as substrates for CtAAOMT and CtFOMT and their monomethyl ethers.

elution condition was gradient elution at 0.25 ml min^{-1} by a linear solvent gradient protocol of MeOH–H_2O containing 0.1% formic acid at t=0 (10:90) to 3 min, then to 90:10 at t=23 min, and held at this ratio for an additional 5 min. The elution condition is hereinafter referred to as Su-A. The MS acquisition was operated in selected ion monitoring mode for negative ions at a dwell time of 1.0 s. The LC-MS analysis showed that any apigenin monomethyl ether isomers were not detected, though analyzed with the aid of their authentic samples, which were prepared or purchased previously (Umezawa et al. 2013). On the other hand, LC-MS analysis of the reaction products from luteolin [the elution condition (Hy-B): a Hydrosphere C18 column (10 cm×2.0 mm, 3 μm, YMC, Kyoto, Japan) with a Hydrosphere C18 guard column (1 cm×2.0 mm, YMC), gradient elution at 0.17 ml min^{-1} by a linear solvent gradient protocol of MeOH–H_2O containing 0.1% formic acid at t=0 (37:63) to 60 min, then to 70:30 at t=80 min] showed the presence of a new peak with a retention time different from that of the substrate. The new peak of m/z 299 was assigned to the $[M–H]^-$ ion of luteolin monomethyl ether. The product was identified as 3′-*O*-methylluteolin (chrysoeriol), because its retention time (t_R, 66.3 min) matched that of chrysoeriol (66.4 min), but not the other regioisomers [t_R: 4′-*O*-methylluteolin (diosmetin), 68.0 min; 5-*O*-methylluteolin, 14.9 min; and 7-*O*-methylluteorin, 75.2 min] (Figure 5). The authentic samples were obtained as follows. Chrysoeriol and diosmetin were purchased from Extrasynthese (Genay, France). 5-*O*-Methylluteolin and 7-*O*-methylluteolin were synthesized from luteolin according to Bouktaib et al. (2002) and Pankajamani and Seshadri (1954), respectively. The structures of the synthesized compounds were confirmed by ^{1}H-nuclear magnetic resonance (NMR) spectra obtained using a JNM-LA400MK FT-NMR system (JEOL Ltd., Akishima, Japan). Chemical shifts and coupling constants (J) were expressed in δ values and Hz, respectively. 5-*O*-Methylluteolin: ^{1}H NMR (acetone-d_6): 3.77 (3H, s, OCH_3), 6.31 (1H, s, 3-H), 6.37 (1H, d, J 1.6, 6-H or 8H), 6.52 (1H, d, J 2.0, 8-H or 6H), 6.90 (1H, d, J 8.4, 5′-H), 7.31 (1H, dd, J 2.0, 8.4, 6′-H), 7.37 (1H, d, J 2.0, 2′-H). 7-*O*-Methylluteolin: ^{1}H NMR (acetone-d_6): 3.85 (3H, s, OCH_3), 6.23 (1H, d, J 2.4, 6-H or 8H), 6.52 (1H, s, 3-H), 6.62 (1H, d, J 2.8, 8-H or 6H), 6.87 (1H, d, J 8.4, 5′-H), 7.37–7.40 (2H, m, 2′-H, 6′-H). When the lignan matairesinol was used as a substrate in the same manner as the assay with CtMROMT (Umezawa et al. 2013), formation of arctigenin (4′-*O*-methylmatairesinol) was

not observed.

These results clearly indicated that CtAAOMT methylateed luteolin, but not apigenin, regioselectively to give rise to chrysoeriol. On the other hand, diosmetin was detected in the β-glucosidase-treated MeOH extracts from the 15-DAF *C. tinctorius* seeds, although the presence of trace amounts of chysoeriol could not be ruled out. The flavonoid diosmetin was identified by LC-MS analysis to detect *m*/*z* 299 ion [the elution condition referred to as Hy-C: a Hydrosphere C18 column (10 cm×2.0 mm, 3 μm, YMC) with a Hydrosphere C18 guard column (1 cm×2.0 mm, YMC), gradient elution at 0.17 ml min^{-1} by a linear solvent gradient protocol of MeOH–H_2O containing 0.1% formic acid at t=0 (40 : 60) to 35 min, then to 70 : 30 at t=50 min] by comparison of retention time (t_R, 42.7 min) with those of authentic samples (chrysoeriol, 42.1 min; diosmetin, 42.7 min). Figure 4B shows that the flavonoids amounts, which were determined by LC-MS analysis under the elution condition Su-A, increased after 12 DAF.

In contrast to CtAAOMT, CtFOMT methylated apigenin regioselectively to give rise to acacetin (Umezawa et al. 2013). In addition, the expression profile of the gene *CtFOMT* (Figure 4A) (Umezawa et al. 2013) was in good accordance with diosmetin and luteolin accumulation (Figure 4B). However, its luteolin methylation was not yet examined. So, we prepared the recombinant CtFOMT and assayed the activity of recombinant CtFOMT toward luteolin as previously for apigenin methylation (Umezawa et al. 2013). LC-MS analysis of the reaction products to detect *m*/*z* 299 ion was conducted under the elution condition Hy-B, which indicated the formation of diosmetin (t_R, 67.8 min; t_R of authentic sample, 67.9 min). The reaction kinetics of recombinant CtFOMT was determined as the phenylpropanoid monomer assay with CtAAOMT. Briefly, the reaction mixture (total volume 200 μl) contained 50 mM of Tris-HCl (pH 7.5), 2 mM of $MgCl_2$, 200 μM of SAM, substrate [apigenin (0.1–100 μM) or luteolin (0.1–20 μM)], and the appropriate volume of enzyme solution was incubated at 30°C for 20 min and the reaction was terminated by adding 500 μl of ethyl acetate containing naringenin (purchased from Extrasynthese, Genay, France) as internal standard. The ethyl acetate extracts were dried and subjected to LC-MS analysis under the elution condition Su-A. V_{max} and K_m values were determined from Lineweaver–Burk plots. The K_m values of CtFOMT were 2.2 μM for apigenin and 2.5 μM for luteolin, while the k_{cat}/K_m values for CtFOMT were 0.34 μM^{-1} min^{-1} for apigenin and 0.29 μM^{-1} min^{-1} for luteolin.

Apigenin and luteolin methylation activities were also detected in crude enzyme preparations from the seeds. Regioselective apigenin methylation to afford acacetin was detected for enzyme preparations from 12, 15, or 18 DAF seeds, which is in accordance with the time profile of *CtFOMT* transcript (Figure 4A) (Umezawa et al. 2013), while regioselectivity of luteolin methylation varied with the stages of seed maturation; luteolin methylation to afford chrysoeriol (3′-*O*-methylluteolin) (t_R, 66.6 min, t_R of authentic sample, 66.6 min) was detected from the 6 and 9 DAF seeds by LC-MS analysis to detect *m*/*z* 299 ion (the elution condition Hy-B), whereas enzyme preparations from 12, 15, or 18 DAF gave both chrysoeriol and diosmetin (4′-*O*-methylluteolin) (t_R, 67.9 min; t_R of authentic sample, 67.8 min). The time profile of flavonoid 4′-OMT activity during seed maturation was synchronized with the gene expression pattern of *CtFOMT* (Figure 4A). However, the *CtAAOMT* gene expression pattern (Figure 4A) was not synchronized with the accumulation of the flavonoid compounds (Figure 4B). Taken together, these results strongly suggest the involvement of *CtAAOMT* in lignin biosynthesis, but not in flavonoid methylation in *C. tinctorius*, while CtFOMT is probably involved in diosmetin formation from luteolin in addition to acacetin formation from apigenin in the seeds.

Although no OMTs that have high sequence homology with CtAAOMT have been reported (Figure 3), there are many EST clones that have high homology to *CtAAOMT*. When Blast searches of EST databases were carried out on January 20, 2014 using the *CtAAOMT* open reading frame sequence as a query, 40 *OMT* ESTs with high sequence homology to *CtAAOMT* were found (with E-values of less than 2e^{-107} for fragments of more than 50% query cover). Among these *OMT* ESTs were those from other members of the Asteraceae, such as *Centaurea solstitialis* (yellow star thistle), *Helianthus tuberosus* (Jerusalem artichoke), *Helianthus annuus* (common sunflower), *Helianthus paradoxus* (puzzle sunflower), *Taraxacum officinale* (dandelion), *Lactuca saligna* (willowleaf lettuce). These OMT fragments have 77–96% sequence identity to appropriate partial sequences of *CtAAOMT*. These results suggested that *CtAAOMT* genes are structurally conserved in Asteraceae plants.

In conclusion, we have isolated a novel cDNA encoding *O*-methyltransferase (CtAAOMT) that catalyzed 5- and 3-*O*-methylation of hydroxycinnamaldehydes and hydroxycinnamyl alcohols. Biochemical characterization of the OMT and its gene expression analysis strongly suggested that CtAAOMT is involved in biosynthesis of syringyl lignin in addition to CtCAldOMT1.

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