

A lignan *O*-methyltransferase catalyzing the regioselective methylation of matairesinol in *Carthamus tinctorius*

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Abstract Lignans are a group of plant phenolic compounds with various biological activities, including antitumor and antioxidant properties. *O*-Methylation is a critical step in biosynthesis of these compounds. However, little is known about the *O*-methyltransferase (OMT) enzymes that catalyze lignan *O*-methylation. We discovered a highly regioselective OMT activity in safflower (*Carthamus tinctorius*) seeds that catalyzed the methylation of matairesinol, a dibenzylbutyrolactone lignan, into 4'-*O*-methylmatairesinol (arctigenin) but not 4-*O*-methylmatairesinol (isoarctigenin). By examining such OMT activity in correlation with OMT transcript abundances during seed development, we cloned a few putative OMT cDNAs and produced their recombinant proteins in *Escherichia coli*. Among them, one protein exhibited *O*-methylation activity for matairesinol with the regioselectivity identical to that of the plant protein, and was named *C. tinctorius* matairesinol OMT (CtMROMT). CtMROMT did not show any detectable OMT activities towards phenylpropanoid monomers under the reaction conditions tested, while it methylated flavonoid apigenin efficiently into 4'-*O*-methylapigenin (acacetin). However, quantitative real-time polymerase chain reaction analysis demonstrated that expression of the CtMROMT gene was synchronized with the CtMROMT activity profile and arctigenin accumulation in the plant. These results demonstrated that CtMROMT is a novel plant OMT for lignan methylation.

Key words: *Carthamus tinctorius*, lignan, matairesinol, *O*-methyltransferase, regioselective methylation.

In plant secondary metabolic processes, *O*-methylation is usually catalyzed by *S*-adenosyl-L-methionine-dependent *O*-methyltransferases (OMTs), and is crucial in determining final product distribution (Noel et al. 2003; Umezawa 2010). During the last two decades, OMTs involved in lignin biosynthesis and many other plant OMTs catalyzing the methylation of flavonoids, stilbenes, coumarins, phenylpropenes, and alkaloids have been characterized (Ibrahim et al. 1998; Joshi and Chiang 1998; Schröder et al. 2002; Zubieta et al. 2001). On the other hand, genes encoding OMTs for methylation of lignans (lignan OMTs) have not yet been reported, although lignans constitute an abundant class of plant phenolic compounds.

The basic carbon skeleton of lignans is composed of two phenylpropanoid units that are linked by the central carbon (C8) of their side chains (Umezawa

2003a). Lignan structures show diverse oxidation levels and substitution patterns (Umezawa 2003a, 2003b). For example, lignans with various *O*-methylation (methoxylation) patterns are distributed widely in the plant kingdom (Umezawa 2003a, 2003b). These methoxylated lignans have bioactivities that are useful for human health and play important physiological roles in plant metabolism (Harmatha and Dinan 2003; MacRae and Towers 1984; Umezawa 2003a).

A trimethoxylated aryltetralin lignan, podophyllotoxin (Figure 1), is an important antitumor agent that has been used as a raw material to prepare semi-synthetic antitumor medicines for cancer chemotherapy (Srivastava et al. 2005; You 2005). A trimethoxylated dibenzylbutyrolactone lignan, arctigenin (4'-*O*-methylmatairesinol) is another antitumor lignan with selective toxicity against cancer cells under glucose

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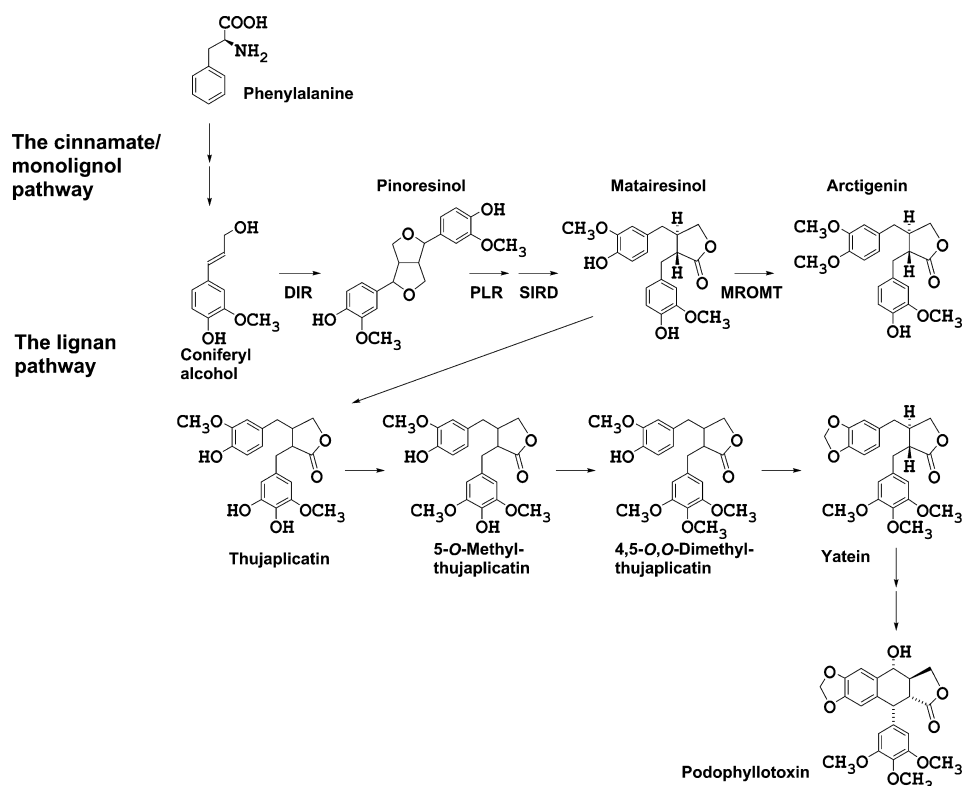


Figure 1. The cinnamate/monolignol pathway and the lignan pathway. DIR: dirigent protein, PLR: pinoresinol/lariciresinol reductase, SIRD: secoisolariciresinol dehydrogenase, MROMT: matairesinol OMT.

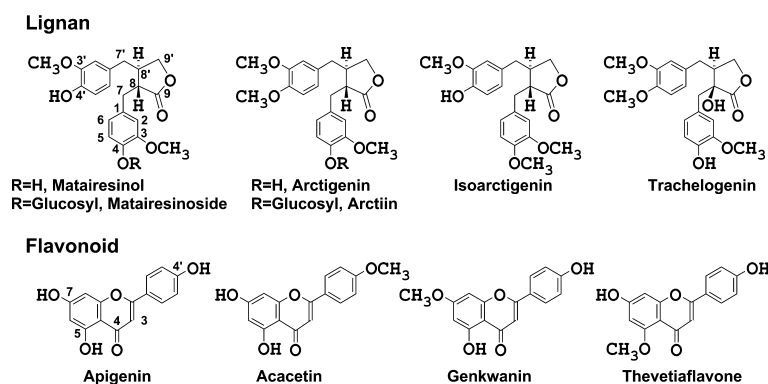


Figure 2. Structures of lignans and flavonoids relevant to the present study.

starvation (Awale et al. 2006; Kim et al. 2010; Sun et al. 2011). Arctigenin also has various other biological functions, such as antiproliferative (Matsumoto et al. 2006; Ryu et al. 1995), hepatoprotective (Kim et al. 2003), anti-inflammatory, and analgesic activities (Fan et al. 2006; Kang et al. 2008). It also suppresses the heat shock response in mammalian cells (Ishihara et al. 2006), inhibits mitogen-activated protein kinases (Cho et al. 2004) and cyclic AMP phosphodiesterase (Nikaido et al. 1981), and has a relaxation effect on histamine-induced contraction of tracheal muscles (Fujimoto et al. 1992).

In relation to the physiological functions of methoxylated lignans in plants, it was suggested that

arctigenin and its acid form, arctigenic acid, play important roles in the stimulatory allelopathy of *Arctium lappa* seeds during the seed germination stage (Higashinakasu et al. 2005). In addition, arctigenin (Chang et al. 1999; Chang et al. 2000) and another trimethoxylated dibenzylbutyrolactone lignan, yatein (Figures 1, 2) (Erdtman and Harmatha 1979) are typical examples of heartwood lignans, which are deposited in the heartwood region of trees and probably prevents heart rot caused by heart-rot fungi (Suzuki et al. 2001; Suzuki et al. 2002a; Suzuki et al. 2004; Suzuki and Umezawa 2007).

Methoxyl groups present in lignan molecules can

be classified into two subgroups on the basis of their biosynthesis: 3(3′)-methoxyl groups and others (Figures 1, 2). The 3(3′)-methoxyl groups are introduced into phenylpropanoid monomers in the cinnamate/monolignol pathway (Umezawa 2010), and are retained in lignan molecules. Many lignans are formed by dimerization of coniferyl alcohol (Suzuki and Umezawa 2007; Umezawa 2003a). The *O*-methyl group of coniferyl alcohol used for lignin biosynthesis is introduced by caffeoyl CoA OMT (CCoAOMT)-catalyzed methylation of caffeoyl CoA (Umezawa 2010). However, it is still an open question whether coniferyl alcohol as the lignan precursor is synthesized by the CCoAOMT isoenzymes involved in lignin biosynthesis, by different CCoAOMT isoenzymes, or even by another OMT. Sakakibara et al. suggested that the coniferyl alcohol used in lignan biosynthesis may be formed via ferulic acid, which may not require CCoAOMT (Sakakibara et al. 2007). The other methoxyl groups in lignans, e.g. the 4(4′), 5(5′), and 6(6′)-methoxyl groups on the aromatic rings, are most probably introduced by lignan-OMT-catalyzed, post-coupling methylation of the corresponding 4(4′), 5(5′), and 6(6′)-hydroxylignans (Suzuki and Umezawa 2007; Umezawa 2003a) (Figures 1, 2). There are only a few reports on enzyme-mediated lignan methylation. Ozawa et al. reported OMT activities of cell-free extracts from *Forsythia intermedia* (var. Lynwood Gold) (Ozawa et al. 1993) and *Magnolia kobus* var. *borealis* (Miyauchi and Ozawa 1998). β -Peltatin 6-*O*-methyltransferase was isolated and characterized from cell suspension cultures of *Linum nodiflorum* by Kranz and Petersen (Kranz and Petersen 2003).

Members of the tribe Cynareae in the family Asteraceae such as *Carthamus tinctorius* (safflower) are enriched with 4′-*O*-methyl dibenzylbutyrolactone lignans such as arctigenin and arctiin (Figure 2), especially in their seeds (Hänsel et al. 1964; Nishibe et al. 1972; Palter and Lundin 1970; Palter et al. 1971; Palter et al. 1972; Sakakibara et al. 2007; Suzuki et al. 2002b; Umezawa 2003b). Our survey of lignans in *C. tinctorius* indicated that matairesinol and arctigenin (or their glucosides) started to accumulate several days after flowering (DAF) (Sakakibara et al. 2007). Here, we report the first molecular cloning and characterization of a lignan OMT cDNA in *C. tinctorius* seeds. The OMT catalyzes monomethylation of matairesinol to give rise to arctigenin, but not its regioisomer, isoarctigenin (4-*O*-methylmatairesinol) (Figure 2), and is designated as *C. tinctorius* matairesinol OMT (CtMROMT).

Materials and methods

Plant materials

Seeds of *C. tinctorius* cv. Round-leaved White were purchased from Takii Seed Co. Ltd. (Kyoto, Japan) and sown in garden

soil. The germinated plants were maintained in a plant incubator (16-h light/8-h dark photoperiod at 25°C) or in a greenhouse facility at Michigan Technological University. Seeds and other organs were snap-frozen in liquid nitrogen immediately after harvest and stored in liquid nitrogen until protein and RNA isolation.

Instrumentation

¹H-NMR spectra were obtained with a JNM-LA400MK FT NMR System (JEOL Ltd., Akishima, Japan). Chemical shifts and coupling constants (*J*) are given in δ and Hz, respectively. The equipments and conditions for GC-MS were as follows: a JMS-DX303HF mass spectrometer equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system (JEOL Ltd.), electron-ionization (EI) mode, 70 eV; gas-chromatographic column, Shimadzu HiCap CBP10-M25-025 (5 m×0.22 mm) (Shimadzu Co., Kyoto, Japan); temperature, 40°C at *t*=0–2 min, then to 230°C at 30°C min^{−1}; carrier gas, He; splitless injection; a Shimadzu GCMS-QP5050A (Shimadzu Co.), EI mode, 70 eV; gas-chromatographic column, Shimadzu HiCap CBP10-M25-025 (25 m×0.22 mm) (Shimadzu Co.); temperature, 40°C at *t*=0–2 min, then to 240°C at 30°C min^{−1}; carrier gas, He; splitless injection; and a Shimadzu GCMS-QP2010 Plus (Shimadzu Co.), EI mode (70 eV); column, Shimadzu HiCap CBP10-M25-025 column (10 m×0.22 mm) (Shimadzu Co.); carrier gas, He; temperature, 160°C at *t*=0–2 min, then to 250°C at 10°C min^{−1}. Samples dissolved in *N,O*-bis(trimethylsilyl)acetamide were subjected to GC-MS measurement after heating at 60°C for 45 min. Reversed phase HPLC-MS for flavonoid analysis was conducted with a system composed of a Shimadzu LC-10ADvp HPLC series liquid chromatograph equipped with a Shimadzu SPD-10Avp UV-Vis detector and a Shimadzu LCMS-2010A single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in positive/negative ion mode (Shimadzu Co.). The LC columns used were as follows. For quantitative analysis of flavonoids formed in plants or in enzyme experiments, a Supelcosil ABZ-Plus column (25 cm×2.1 mm, 5 μ m, Supelco, Bellefonte, PA, USA) which was eluted at 40°C at 0.25 ml min^{−1} by a linear solvent gradient protocol of methanol–H₂O containing 0.1% HCOOH 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. For identification of flavonoids formed in plants or in enzyme experiments, a Phenomenex Gemini 3 μ m C18 110 Å column (15 cm×2.0 mm, 3 μ m) (Phenomenex, Torrance, CA, USA) was eluted at 40°C at 0.12 ml min^{−1} by an initial isocratic elution followed by two linear solvent gradient protocols of solvent A [H₂O–methanol, (85:15), containing 0.1% HCOOH] and solvent B [acetonitrile–methanol, (85:15), containing 0.1% HCOOH] at *t*=0 (85:15) to 5.0 min, then to 40:60 at *t*=35.0 min, further to 0:100 at *t*=37.0 min, and this ratio was held for an additional 3 min.

Chemical synthesis and chemicals

(\pm)-Matairesinol (Umezawa et al. 1992) and (\pm)-[3-OC²H₃]-matairesinol (Sakakibara et al. 2003) were prepared previously. (\pm)-Arctigenins were prepared as previously reported (Sakakibara et al. 2003; Sakakibara et al. 2007; Umezawa et al. 1992); ¹H-NMR δ_{H} (CDCl₃) 2.45–2.63 (4H, m), 2.88 (1H, dd, *J* 6.56 and 14.08), 2.93 (1H, dd, *J* 5.36 and 14.08), 3.80 (6H, s), 3.83 (3H, s), 3.86 (1H, dd, *J* 4.40 and 7.08), 4.12 (1H, dd, *J* 7.08 and 9.04), 6.44 (1H, d, *J* 1.96), 6.53 (1H, dd, *J* 1.96 and 8.08), 6.59 (1H, dd, *J* 1.94, 8.06), 6.62 (1H, d, *J* 1.96), 6.73 (1H, d, *J* 8.00), 6.80 (1H, d, *J* 8.00); δ_{H} (CD₃COCD₃) 2.53–2.69 (4H, m), 2.83 (1H, dd, *J* 6.72 and 14.04), 2.93 (1H, dd, *J* 5.19 and 13.89), 3.76 (3H, s), 3.76 (3H, s), 3.80 (3H, s), 3.90 (1H, dd, *J* 7.94 and 8.85), 4.10 (1H, dd, *J* 7.17 and 9.00), 6.64 (1H, dd, *J* 2.14 and 8.24), 6.68 (1H, dd, *J* 1.83 and 7.94), 6.69 (1H, d, *J* 2.14), 6.76 (1H, d, *J* 7.94), 6.82 (1H, d, *J* 1.83), 6.83 (1H, d, *J* 8.24). (\pm)-Isoarctigenins were prepared in the same manner as (\pm)-matairesinol and (\pm)-arctigenins, but with (\pm)-3-(4-benzyloxy-3-methoxybenzyl)- γ -butyrolactones and veratraldehyde; ¹H-NMR δ_{H} (CDCl₃) 2.45–2.63 (4H, m), 2.89 (1H, dd, *J* 6.84 and 14.16), 2.96 (1H, dd, *J* 5.24 and 14.04), 3.80 (3H, s), 3.81 (3H, s), 3.84 (3H, s), 3.86 (1H, dd, *J* 7.32 and 9.00), 4.12 (1H, dd, *J* 7.08 and 9.04), 6.42 (1H, d, *J* 1.96), 6.50 (1H, dd, *J* 1.82 and 7.94), 6.63–6.66 (2H, m), 6.75 (1H, d, *J* 8.80), 6.79 (1H, d, *J* 8.04); δ_{H} (CD₃COCD₃) 2.51–2.71 (4H, m), 2.85 (1H, dd, *J* 7.02 and 14.04), 2.94 (1H, dd, *J* 5.34 and 13.89), 3.77 (3H, s), 3.77 (3H, s), 3.79 (3H, s), 3.89 (1H, dd, *J* 8.09 and 9.00), 4.10

(1H, dd, *J* 7.02 and 9.16), 6.58 (1H, dd, *J* 1.98, 8.09), 6.69 (1H, d, *J* 1.83), 6.72 (1H, d, *J* 7.94), 6.75 (1H, dd, *J* 2.14, 8.24), 6.84 (1H, d, *J* 2.14), 6.86 (1H, d, *J* 8.24). GC-MS data for the arctigenin and isoarctigenin (TMS ethers) thus synthesized are shown in the Results (Figure 3).

A mixture of (\pm)-[4'-OC²H₃]arctigenins and (\pm)-[4-OC²H₃]isoarctigenins was prepared by deuteromethylation of (\pm)-matairesinol with C²H₃I (99.5+at% ²H, Aldrich Chem. Co., Milwaukee, WI, USA). The ratio of the two isomers was determined to be 1:1 by comparing intensities of the doublet of doublet signals [δ_{H} (CD₃COCD₃) 6.64 (arctigenin); 6.58 (isoarctigenin)] in the ¹H-NMR spectra. GC-MS data for the deuterium-labeled arctigenin and isoarctigenin (TMS ethers) thus synthesized are shown in the Results (Figure 8).

Acacetin (4'-O-methylapigenin) was purchased from ICN Biomedicals (Irvine, CA, USA), genkwanin (7-O-methylapigenin) from Extrasynthese (Genay, France), and apigenin from Wako Pure Chemical Industries (Osaka, Japan). Thevetiaflavone (5-O-methylapigenin) was synthesized from apigenin according to Bouktaib et al. (2002). Thevetiaflavone; δ_{H} (CD₃COCD₃): 3.77 (3H, s), 6.36 (1H, s), 6.37 (1H, d, *J* 2.4), 6.54 (1H, d, *J* 2.4), 6.93 (2H, d, *J* 8.8), 7.79 (2H, d, *J* 6.8); ESI-MS (positive): *m/z* 285 ([M+H]⁺).

Matairesinol O-methylation by plant proteins extracted from *C. tinctorius* seeds

C. tinctorius seeds (approx. 0.5 g) were pulverized in liquid nitrogen with a mortar and pestle and homogenized with the mortar and pestle on ice for approximately 5 min in 1.75 ml extraction buffer (0.1 M potassium phosphate, pH 7.5) containing 0.01 M dithiothreitol, 0.1 g polyvinylpyrrolidone (polyclar AT), and 0.1 g sea sand. The homogenate was transferred to two 2.0 ml-microcentrifuge tubes and centrifuged (10,000 $\times g$ for 15 min at 4°C). The supernatant was filtered through a membrane filter (Millipore Millex-GX, pore size, 0.22 μ m, Millipore, Carrigtwohill, Co. Cork, Ireland). The filtrate (approx. 1.0 ml) was passed through a Sephadex G-25 Coarse gel column (GE Healthcare Bio-Science AB, Uppsala, Sweden) preequilibrated and eluted with 50 mM Tris-HCl (pH 8.0) at 4°C. The excluded fraction (ca. 1.2 ml) was used immediately for the OMT activity methylating matairesinol (matairesinol OMT, MROMT) assay. The basic reaction mixture contained 38.4 μ l 50 mM Tris-HCl buffer (pH 8.0), 20 μ l 20 mM MgCl₂ in 50 mM Tris-HCl buffer (pH 8.0), 120 μ l enzyme preparation, 1.6 μ l 5 mM S-adenosyl-L-methionine (SAM) in H₂O, and 20 μ l 1 mM (\pm)-[3-OC²H₃]matairesinol in a mixture of H₂O and methanol (1:1). The reaction was initiated by adding the enzyme preparation, and incubated at 30°C for 60 min. The reaction was terminated by extracting with ethyl acetate containing 0.10 μ g each (\pm)-arctigenins and (\pm)-isoarctigenins. The solvent was removed from ethyl acetate solutions under high vacuum. The reaction products thus obtained were analyzed by GC-MS using a Shimadzu QP-2010 plus GC-MS system after trimethylsilylation.

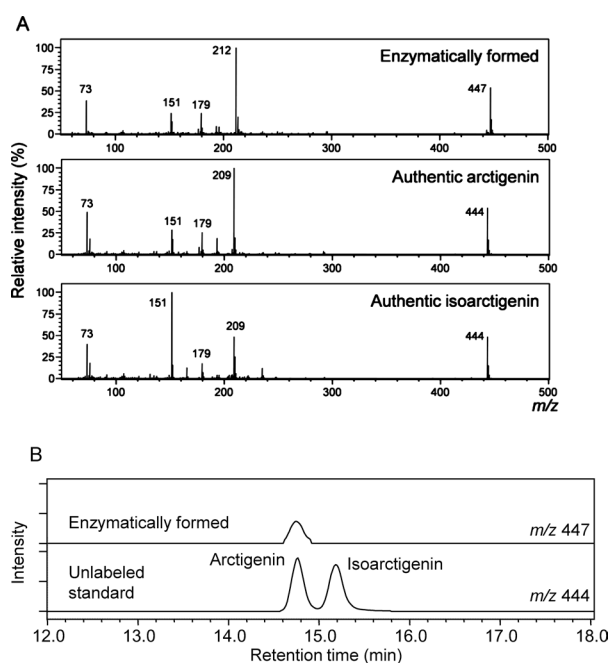


Figure 3. GC-Mass spectra (A) and chromatograms (B) of arctigenin and isoarctigenin (TMS ethers). Enzymatically formed: Formed following incubation of (\pm)-[3-OC²H₃]matairesinol with recombinant CtOMT2 (CtMROMT). Authentic arctigenin and Authentic isoarctigenin: Authentic (\pm)-arctigenins and (\pm)-isoarctigenins, respectively. Unlabeled standard: (\pm)-Arctigenins (Arctigenin) and (\pm)-isoarctigenins (Isoarctigenin) as internal standards.

Detection and quantitation of flavonoids in *C. tinctorius* seeds

Methanol extraction of *C. tinctorius* seeds was conducted as previously reported (Sakakibara et al. 2007). Briefly, the seeds were frozen in liquid N₂, freeze-dried, powdered, and extracted with hot methanol. The methanol solution was extracted with hexane and the solvent, methanol, was evaporated off to dryness. The methanol extracts thus obtained were further treated with β -glucosidase (Sigma-Aldrich G-0395, 1480 U/g MeOH extracts, Sigma-Aldrich, Saint Louis, MO, USA) in sodium acetate buffer (pH 5.0) for 24 h at 37°C. The reaction mixture was acidified with 2 N HCl and extracted with ethyl acetate, and the solvent was evaporated off. The extracts were dissolved in methanol, and then, filtered and analyzed by LC-MS to identify flavonoids. To quantitate flavonoids, methanol extracts were prepared as described above except that the hexane extraction step was included for powdered seeds, but not for the methanol solution. The methanol extracts were analyzed by LC-MS after addition of [OC²H₃]ferulic acid as an internal standard.

Cloning of cDNAs encoding *C. tinctorius* OMTs

Total RNA was isolated from *C. tinctorius* seeds collected at 12 DAF following the method of Bugos et al. (Bugos et al. 1995). Poly(A)⁺ RNA was obtained from the total RNA and used to construct a cDNA library in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The cDNA library was screened using a *Populus tremuloides* (aspen) CALdOMT-encoding cDNA (*PtreOMT1*) (Bugos et al. 1991; Li et al. 2000) as a probe, and a full-length cDNA was cloned and sequenced (GenBank AB430466). This cDNA exhibited 72.8% amino acid sequence identity with *PtreOMT1*, and its recombinant protein showed CALdOMT activity, but not matairesinol methylation activity. Hence, the cDNA was designated as *CtCALdOMT1* (Nakatsubo et al. 2007).

Then, lambda-phage cDNAs were isolated from the cDNA library according to reference (Ausubel et al. 1990) and used as a template for polymerase chain reaction (PCR) amplification of OMT cDNAs. The open reading frame sequence of

CtCALdOMT1 was used for BLAST searches and 40 OMT genes showing highest homology to *CtCALdOMT1* were selected. In addition, to cover various OMTs belonging to the PI-OMT II subclass, seven plant OMT genes reported previously (NDong et al. 2003) were also selected. Because the plant protein assay showed that *C. tinctorius* MROMT depended on SAM for its activity (Table 1), we compared codon usage in motif A (Joshi and Chiang 1998) among the 47 genes. Based on this analysis, we designed the degenerate primer 5'-TCYDTKGTTGATGTTGGTGG-3'. The primer and an oligo dT primer were used for PCR. The amplified cDNAs were ligated into pCR2.1-TOPO or pCR2.1 vectors and transformed into *Escherichia coli* TOP10F' (Invitrogen Life Technologies Corp., Carlsbad, CA, USA). Then, 215 randomly selected colonies were submitted to direct colony PCR using M13 forward and reverse primers, and 32 independent cDNAs with inserts of approximately 0.2–1 kb were selected. Sequencing of the 32 cDNAs classified them into four separate groups, three of which (*CtO1*, *CtO2*, and *CtO3*) could be assigned to OMT gene family members. *CtO1* was a fragment of *CtCALdOMT1* (Nakatsubo et al. 2007). *CtO2* and *CtO3* were used to screen the cDNA library to obtain the corresponding full-length cDNAs, designated as *CtOMT2* and *CtOMT3* [DNA Data Bank of Japan (DDBJ) accession number, AB741899 and AB741900, respectively].

Quantitative real-time PCR analysis

Quantitative real-time PCR was used for expression analysis of *CtOMT2* and 3. Total RNAs were extracted from *C. tinctorius* using the Plant RNeasy extraction kit (Qiagen GmbH, Hilden, Germany) or the method of Bugos et al. (Bugos et al. 1995). The RNA concentration was estimated by measuring its absorbance at 260 nm. An aliquot of the RNA extracts was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies Corp.) and random hexamers, according to the manufacturer's protocol. Quantitative real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems LLC, Foster City, CA, USA). The total volume of the reaction mixture was 50 μ l containing 1 μ l cDNAs, 200 nM gene-specific primers and 25 μ l 2 \times SYBR Green Universal Master Mix (Applied Biosystems LLC), with a preliminary step of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To check the specificity of annealing of the oligonucleotides, dissociation kinetics was performed by the machine at the end of the experiment. For quantitation, standard curves were constructed using each recombinant plasmid containing full-length sequences. The 18S ribosomal RNA was analyzed as an internal standard to normalize the transcript abundance in each sample.

Expression of *CtOMT2* and 3 in *E. coli*

PCR was used to introduce an *Nde*I site at the 5'-end and a *Not*I site at the 3'-end of the coding sequence of the *CtOMTs* using sense primers (*CtOMT2*, 5'-TCA TAT GAG TAC ATT TAC AGG AGA -3'; *CtOMT3*, 5'-TCA TAT GGG TAG TAC ATC TAC GAT -3') and antisense primers (*CtOMT2*, 5'-TGC GGC

Table 1. Formation of arctigenin by enzyme preparation from *C. tinctorius* seeds.

Assay ^a	Component ^b	Arctigenin formation ^c
Complete ^d	MR- <i>d</i> ₃ /SAM	0.529 \pm 0.026
	MR- <i>d</i> ₃ /SAM/Mg ²⁺	0.514 \pm 0.003
Control ^d	MR- <i>d</i> ₃	0.027 \pm 0.00009
	SAM	0.008 \pm 0.00012
	Denatured enzyme/ MR- <i>d</i> ₃ /SAM	0.009 \pm 0.002

^aThe assay used a mixture of 12, 14, and 15 DAF seeds. ^bMR-*d*₃: (\pm)-[3-OC²H₃]matairesinols. SAM: S-adenosyl-L-methionine. ^cExpressed in nmol h⁻¹ mg⁻¹ protein. Mean \pm standard deviation ($n=3$). ^dComplete assay mixtures include MR-*d*₃ and SAM with and without Mg²⁺. Control experiments refer to assay lacking either SAM or MR-*d*₃ or complete assay with denatured enzyme (boiled for 5 min).

CGCATCTGAATAGACTTTCGATGA-3'; *CtOMT3*, 5'-TGC GGCCGCATCTGGATAAGCTTTCGATGA-3'). The PCR products were first cloned into pCR2.1 vectors (Invitrogen Life Technologies Corp.), from which the *Nde*I and *Not*I fragments containing the *OMT* coding regions were then cloned into pET23a expression vectors (Novagen, San Diego, CA, USA) to fuse a His-tag at the C-terminal of the cDNA expression product. After sequencing to confirm the accuracy of the *CtOMT*-pET23a constructs, each construct was transferred into *E. coli* BL21 (DE3) (Novagen). The induction and expression of recombinant *CtOMT2* and 3 were conducted according to Li et al. (Li et al. 2000; Li et al. 2001). The BL21 (DE3) cell strain containing the pET23a vector without the *OMT* cDNA insert was used as a control. After harvesting by centrifugation ($2,000 \times g$ for 10 min), the cell pellet was processed to purify the *OMT* proteins using the HisBind Resin (Novagen) according to the manufacturer's protocol.

OMT assay and reaction kinetics of recombinant *C. tinctorius OMT2* and 3 proteins

The basic reaction mixture contained 118.4 μ l 50 mM Tris-HCl buffer (pH 8.0), 60 μ l enzyme preparation in 50 mM Tris-HCl buffer (pH 7.5), 1.6 μ l 5 mM SAM in H_2O , and 20 μ l 1 mM (\pm)-matairesinol in a mixture of H_2O and methanol (1:1). An assay with the same reaction mixture but with 98.4 μ l 50 mM Tris-HCl buffer (pH 8.0) and 20 μ l 20 mM $MgCl_2$ in 50 mM Tris-HCl buffer (pH 8.0) instead of 118.4 μ l 50 mM Tris-HCl buffer (pH 8.0) was also tested. The reaction was initiated by adding the enzyme preparation, and then incubated at 30°C for 60 min. The reaction was terminated by adding ethyl acetate containing 0.30 μ g each of (\pm)-[4'- OC^2H_3]arctigenins and (\pm)-[4- OC^2H_3]isoarctigenins. The ethyl acetate solutions were washed with a saturated NaCl solution and dried over anhydrous Na_2SO_4 . After evaporation to dryness, the extracts were analyzed by GC-MS using the Shimadzu GCMS QP-5050A after trimethylsilylation.

To characterize the enzyme pH optimum, the substrates and recombinant *CtOMT2* protein were used at the concentrations described above in assay mixtures with pHs ranging from 6.0 to 9.0. The buffers were 50 mM potassium phosphate (pH 6.0–8.0) and 50 mM Tris-HCl (pH 7.5–9.0). For kinetics, varying concentrations of (\pm)-matairesinol (2.5 to 100 μ M) and SAM (2 to 200 μ M) were used to measure K_m , V_{max} , and the enzyme turnover number, k_{cat} . All reactions were terminated by adding ethyl acetate containing 0.20 μ g each of (\pm)-[4'- OC^2H_3]arctigenins and (\pm)-[4- OC^2H_3]isoarctigenins. The reaction products were analyzed by GC-MS as described above. To analyze substrate specificity, lignans, phenylpropanoid monomers, and apigenin were used instead of (\pm)-matairesinol and assayed in the same manner as described above or as previously reported (Nakatsubo et al. 2007).

Results

OMT activity methylating matairesinol in *C. tinctorius* seeds

Incubation of deuterium-labeled (\pm)-[3- OC^2H_3]-matairesinol with the cell-free extracts from 15 DAF seeds of *C. tinctorius* in the presence of SAM yielded a [OC^2H_3]monomethylmatairesinol (Figure 3). GC-MS analysis demonstrated that the [OC^2H_3]monomethylmatairesinol was [OC^2H_3]arctigenin, but not [OC^2H_3]isoarctigenin. The compound was identified by comparing the retention time (t_R , 14.70 min) of the molecular ion (m/z 447) (Figure 3B) and the mass spectra (TMS ether) (Figure 3A) with those of unlabeled authentic samples (TMS ethers) [(\pm)-arctigenins: t_R , 14.74 min (Figure 3B) and the mass spectrum: Figure 3A; (\pm)-isoarctigenins: t_R , 15.20 min (Figure 3B) and the mass spectrum: Figure 3A]. The slightly shorter t_R of deuterium-labeled [OC^2H_3]arctigenin compared with

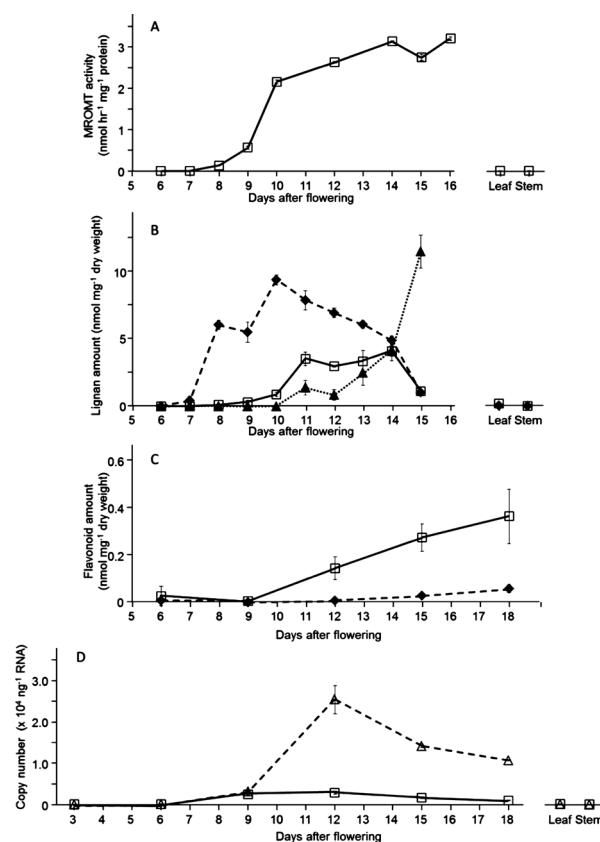


Figure 4. Time course of MROMT activity, accumulation of lignans and flavonoids, and expression profiles of *CtOMT2* and *CtOMT3* in *C. tinctorius*. A: Matairesinol OMT activity in *C. tinctorius* seeds, leaves, and stems; B: Lignan accumulation in *C. tinctorius* seeds (Sakakibara et al. 2007), leaves, and stems (solid diamond, matairesinol; open square, arctigenin; and solid triangle, trachelogenin); C: Flavonoid accumulation in *C. tinctorius* seeds (open square, acacetin; solid diamond, apigenin). D: Expression of *CtOMT2* and *CtOMT3* in *C. tinctorius* seeds, leaves, and stems [open square: *CtOMT2* (*CtMROMT*), open triangle: *CtOMT3* (*CtFOMT*)]. Error bar represents standard deviation [number of replicates (n)=3].

that of the unlabeled standard was due to an isotope effect in GC (Matucha et al. 1991). Control experiments were conducted using the cell-free extracts from 12, 14, and 15 DAF seeds (Table 1). When either the substrate $\{(\pm)\text{-}[3\text{-OC}^2\text{H}_3]\text{matairesinol}\}$ or the cofactor (SAM) was omitted or when denatured enzyme (boiled for 5 min) was used, there was no significant formation of arctigenin. Thus, the plant protein exhibited OMT activity for matairesinol (MROMT activity). The addition of Mg^{2+} to the reaction mixture did not affect its arctigenin-forming activity. As shown in Figure 4A, the MROMT activity in the maturing seeds of *C. tinctorius* appeared on 8 DAF, and high activity was retained after 10 DAF. This time profile was similar to the accumulation peak maxima of arctigenin in the seeds, which was reported previously (Sakakibara et al. 2007) and shown in Figure 4B for comparison. The reduction of arctigenin amounts in the seeds after 14 DAF may be due to its

further metabolism to trachelogenin (Figure 4B).

Accumulation of flavonoids in *C. tinctorius* seeds

In addition to lignans (Sakakibara et al. 2007), we detected the flavonoid apigenin and its monomethyl ether in the methanol extracts from 18 DAF seeds of *C. tinctorius* after β -glucosidase treatment. Apigenin [(I) in Figure 5B] was identified by comparing the mass spectrum ($[\text{M}+\text{H}]^+$, m/z 271, Figure 5A) and t_R (26.07 min) in the LC chromatogram (Figure 5B) with those of authentic apigenin {MS, $[\text{M}+\text{H}]^+$, m/z 271, Figure 5A; t_R , 26.06 min (Figure 5B)}. The monomethyl ether [(II) in Figure 5B] was identified as acacetin, but not the regioisomer, genkwanin or thevetiaflavone, by comparing the mass spectrum ($[\text{M}+\text{H}]^+$, m/z 285, Figure 5A) and t_R (33.84 min) in the LC chromatogram (Figure 5B) with those of all the regioisomers {acacetin: $[\text{M}+\text{H}]^+$, m/z 285 (Figure 5A), t_R , 33.90 min (Figure 5B); genkwanin: t_R , 34.39 min (Figure 5B); and thevetiaflavone: t_R , 16.45 min (Figure 5B)}. This is consistent with previous reports on the isolation of acacetin and its glycoside from the seeds of this plant (Sakamura et al. 1980; Kang et al. 1999). Figure 4C shows the accumulation of the flavonoids during seed maturation.

CtOMT cDNA cloning

MROMT activity coincided with accumulation of its product, arctigenin, in the maturing seeds, leaves and stems of *C. tinctorius* (Figure 4A, B). Therefore, we conducted northern blot analysis using total RNAs isolated from seeds obtained at 6, 9, and 12 DAF. The total RNAs were hybridized under low-stringency conditions with cDNA fragments containing SAM-binding motifs of *PtreOMT1* (GenBank X62096), *Pinus taeda* (loblolly pine) CCoAOMT cDNA (*PtaeCCoAOMT*, GenBank AF036095), and *P. taeda* caffeic acid and caffeoyl CoA ester OMT (AEOMT) cDNA (*PtaeAEOMT*, GenBank U39301). The *PtaeAEOMT* probe did not give strong signals, while the *PtreOMT1* and *PtaeCCoAOMT* probes gave intense positive signals at approximately 1.3 and 1.0 kb, respectively, which decreased with increasing seed maturity. The positive signals could be assigned to *C. tinctorius* 5-hydroxyconiferylaldehyde OMT (*CalDOMT*) and CCoAOMT, respectively. In addition, the *PtreOMT1* probe gave a strong signal at approximately 1.0 kb which increased significantly with increasing seed maturity, suggesting that the signal might correspond to *C. tinctorius* MROMT and that the probe might be useful to screen for a cDNA encoding the MROMT (data not shown). However, screening of the *C. tinctorius* seed cDNA library under the conditions similar to those used in the northern blot experiment always resulted in isolation of a single OMT-like clone (GenBank AB430466). This cDNA

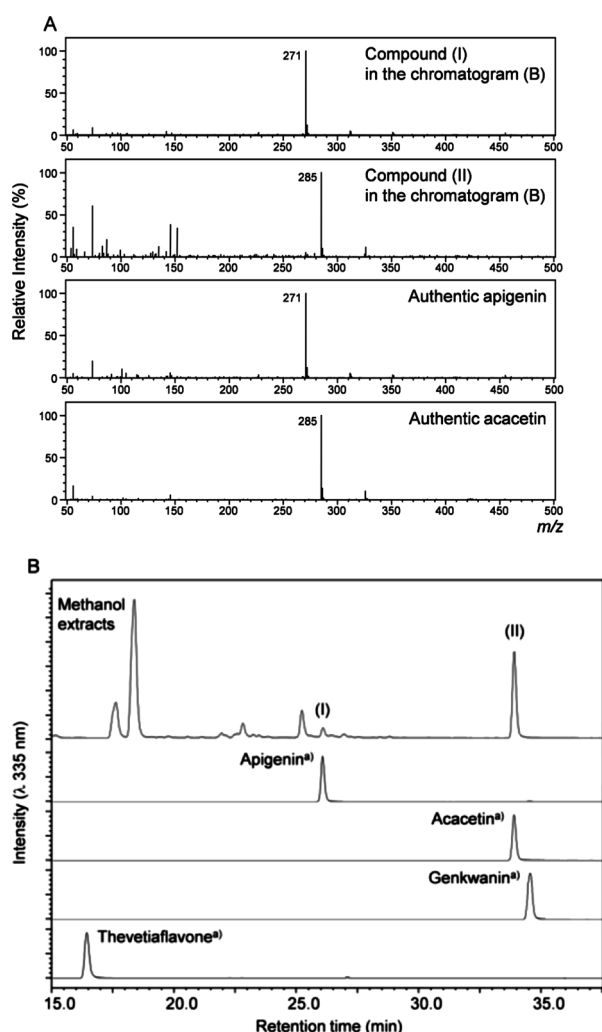


Figure 5. LC-Mass spectra (A) and UV chromatograms (B) of flavonoids. A: Mass spectra (positive ion mode) of compounds (I) and (II) in the chromatogram (B) and authentic flavonoids. B: Chromatograms of the methanol extracts of *C. tinctorius* seeds after β -glucosidase treatment and authentic flavonoids indicated in.^{a)}

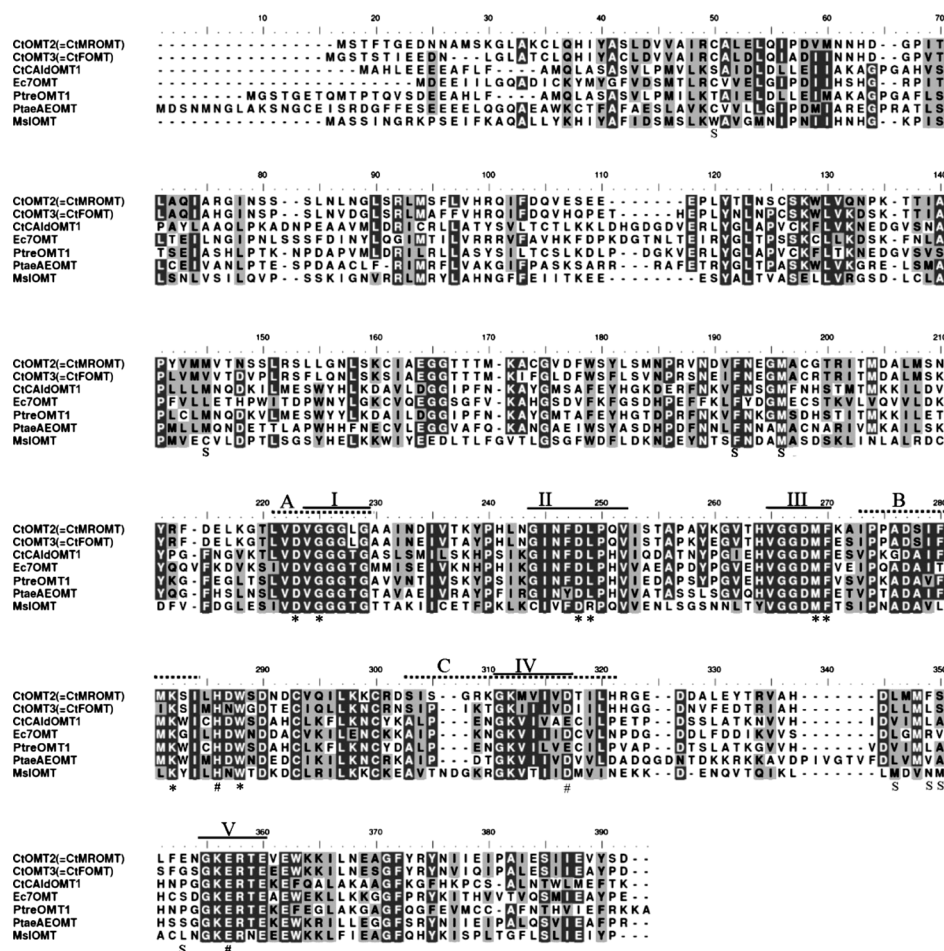


Figure 6. Alignment of the predicted amino acid sequences of CtOMT2 (CtMROMT), CtOMT3 (CtFOMT), CtCaldOMT1, and related OMTs. SAM-binding motifs A, B, and C (Joshi and Chiang 1998); and regions I, II, III, IV, and V are domains conserved among plant OMTs (Dunlevy et al. 2010). #, Catalytic residues; *, SAM binding residues; S, Substrate binding residues of *Medicago sativa* isoflavone OMT (MsiOMT, AAC49927) as determined by Zubieta et al. (2001).

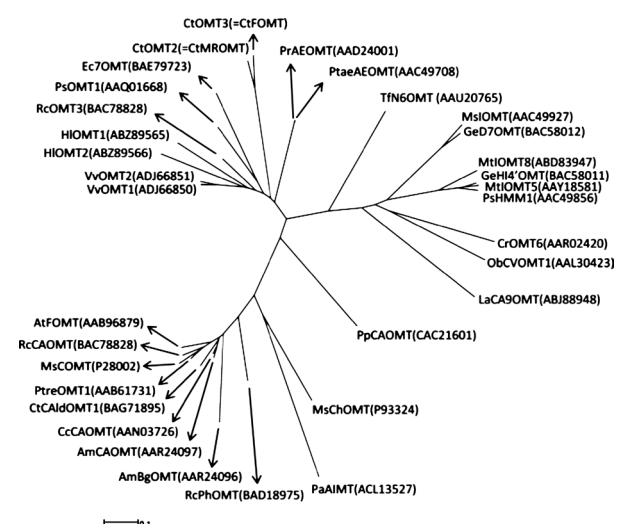


Figure 7. Phylogenetic analysis of CtOMT2 (CtMROMT), CtOMT3 (CtFOMT), and related OMT proteins. GenBank accession numbers are shown in parentheses beside each name.

exhibited 72.8% amino acid sequence identity with *PtreOMT1* (Figures 6, 7) and was identified as *CtCaldOMT1* after kinetic analysis of its recombinant protein (Nakatsubo et al. 2007). However, the recombinant protein did not show MROMT activity.

We then tried a PCR-based strategy using a degenerate primer designed based on codon usage of OMT motif A (Joshi and Chiang 1998) and an oligo dT primer. Three putative OMT cDNA fragments, *CtO1*, *CtO2*, and *CtO3*, were amplified using cDNAs isolated from the cDNA library as a template. Sequencing showed that *CtO1* was a fragment of *CtCaldOMT1*. *CtO2* and *CtO3* appeared to be novel, and therefore, they were used to screen the cDNA library to obtain the corresponding full-length cDNAs, which were designated as *CtOMT2* and *CtOMT3*, respectively.

Sequence analysis of CtOMTs

Both *CtOMT2* and *CtOMT3* cDNAs encode polypeptides of 351 amino acid residues with a calculated molecular mass of 39 kDa (CtOMT2 and 3) and predicted pI values

of 5.21 (CtOMT2) and 5.07 (CtOMT3). Figure 6 shows the amino acid sequences of CtOMT2, CtOMT3, and other selected OMTs. The sequences of the CtOMTs were aligned with those of other plant OMTs using the E-INS-i method in the software “multiple alignment using fast Fourier transform” (MAFFT) 5.0 (Katoh et al. 2005), and were visualized by Bioedit (Hall 1999). The 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 CtOMT2 and CtOMT3 (Figure 6). A phylogenetic tree (Figure 7) was created by the neighbor-joining method using MAFFT 5.0 (Katoh et al. 2005). The phylogenetic tree was viewed and edited using Dendroscope (Huson et al. 2007). Phylogenetic analysis indicated that CtOMT2 and CtOMT3 formed a small clade with caffeic acid *O*-methyltransferase from *Rosa chinensis* var. *spontanea* (RcOMT3) (Wu et al. 2003), reticuline 7-*O*-methyltransferase from *Papaver somniferum* (PsOMT1) (Ounaroon et al. 2003), methoxypyrazine-forming *O*-methyltransferases from *Vitis vinifera* (VvOMT1 and VvOMT2) (Dunlevy et al. 2010), *O*-methyltransferases from *Humulus lupulus* (HlOMT1 and HlOMT2) (Nagel et al. 2008), *Eschscholzia californica* reticuline-7-*O*-methyltransferase (Ec7OMT) (Fujii et al. 2007), and two coniferous AEOMTs (Li et al. 1997; Moyle et al. 2002) (Figure 7). The clade of CtOMT2 and CtOMT3 was separate from that of CALdOMTs [caffeic acid OMTs (CAOMTs)] including PtreOMT1 and CtCALdOMT1 (Figure 7). CtOMT2 and CtOMT3 showed 73.7% amino acid sequence identity with each other.

Expression of CtOMT2 and CtOMT3 mRNAs in *C. tinctorius* seeds

The expression profiles of *CtOMT2* and *CtOMT3* mRNAs during seed maturation and in leaf and stem tissues are shown in Figure 4D. The expression of *CtOMT2* in the seeds was negligible up to 6 DAF and reached its maximum level from 9 to 12 DAF, while its expression in the leaf and stem tissues was negligible. The spatiotemporal expression pattern was consistent with that of MROMT activity in the plant protein extracts (Figure 4A). Compared with *CtOMT2*, *CtOMT3* showed a higher and slightly later expression maximum at 12 DAF (Figure 4D).

Substrate specificity of recombinant CtOMT2 and CtOMT3

We assayed MROMT activity of the recombinant CtOMT2 and CtOMT3. GC-MS analysis of the reaction products indicated that CtOMT2 catalyzed monomethylation of matairesinol, giving rise to arctigenin, but not isoarctigenin (Figure 8). The mass spectrum of the TMS ether of the unlabeled reaction product (Figure 8A) was consistent with the mass

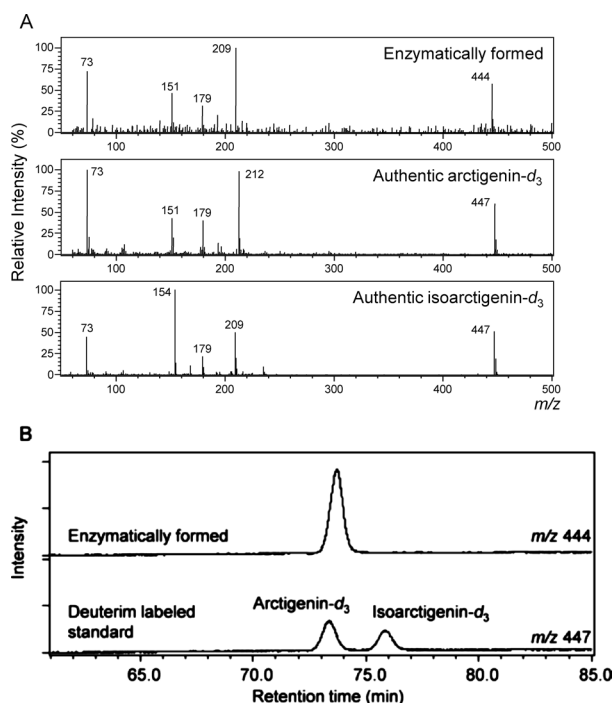


Figure 8. GC-Mass spectra (A) and selected ion monitoring chromatograms (B) of arctigenin and isoarctigenin (TMS ethers). Enzymatically formed: Formed following incubation of (\pm)-matairesinol with recombinant CtOMT2 (CtMROMT). Authentic arctigenin- d_3 and Authentic isoarctigenin- d_3 : Authentic (\pm)-[4'-OC 2 H $_3$]arctigenins and (\pm)-[4-OC 2 H $_3$]isoarctigenins, respectively. Deuterium labeled standard: (\pm)-[4'-OC 2 H $_3$]arctigenins (Arctigenin- d_3) and (\pm)-[4-OC 2 H $_3$]isoarctigenins (Isoarctigenin- d_3) as internal standards.

spectrum of authentic (\pm)-[4'-OC 2 H $_3$]arctigenins, but not (\pm)-[4-OC 2 H $_3$]isoarctigenins (Figure 8A). As can be seen in the selected-ion monitoring (SIM) chromatograms of the TMS ethers of the reaction product and authentic samples (Figure 8B), the t_R of the enzymatically formed methylation product was almost the same as, but slightly larger than, that of the deuterium-labeled standard, (\pm)-[4'-OC 2 H $_3$]arctigenins. This difference was ascribed to the well-known isotope effect on GC t_R (Matucha et al. 1991). On the other hand, the t_R was very different from that of (\pm)-[4-OC 2 H $_3$]isoarctigenins. The methylation activity did not require Mg $^{2+}$, while lysate of the *E. coli* strain containing the pET23a vector without the OMT cDNA insert did not exhibit MROMT activity. Taken together, these results indicate that the recombinant CtOMT2 has regioselective *O*-methylation activity for matairesinol giving rise to arctigenin. Therefore, CtOMT2 was designated as *C. tinctorius* matairesinol OMT (CtMROMT).

Table 2 summarizes the relative activities of recombinant CtMROMT with various substrates. There was no methylation activity against thujaplicatin and 5-*O*-methylthujaplicatin. In addition, CtMROMT did not methylate the phenylpropanoid monomers of the cinnamate/monolignol pathway; there were no

Table 2. Specific activity of recombinant CtOMT2 (CtMROMT) for various substrates.

Substrate	Relative activity (%)
Matairesinol	100
Thuajaplicatin	ND*
5-O-Methylthuajaplicatin	ND*
Caffeic acid	ND**
5-Hydroxyferulic acid	ND**
Caffeoyl CoA	ND**
5-Hydroxyferuloyl CoA	ND**
Caffealdehyde	ND**
5-Hydroxyconiferaldehyde	ND**
Caffeyl alcohol	ND**
5-Hydroxyconiferyl alcohol	ND**
Apigenin	124±74***

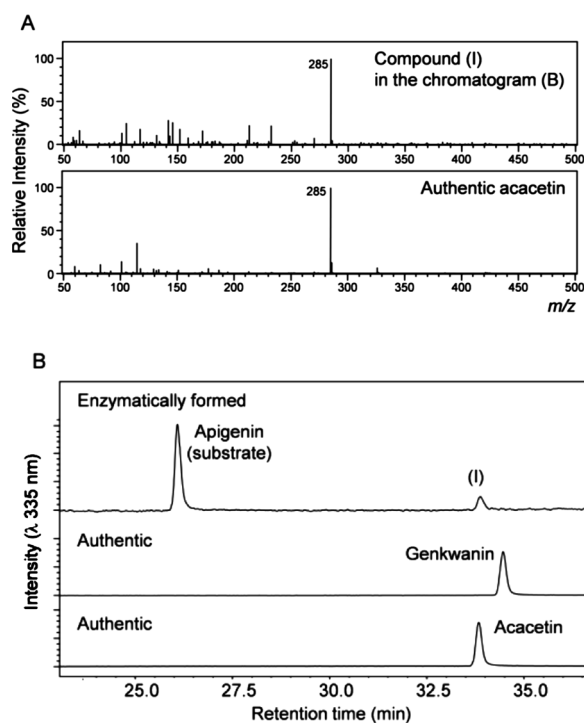
ND: Not detected *: Monomethyl ether formation, $n=2$, **: $n=3$.***: Acacetin formation activity, mean±standard deviation ($n=4$).

Figure 9. LC-Mass spectra (A) and UV chromatograms (B) of flavonoids. A: Mass spectra (positive ion mode) of compound (I) in the chromatogram (B) obtained following incubation of apigenin with recombinant CtOMT2 (CtMROMT) and authentic acacetin. B: Chromatograms of the enzymatic reaction product and authentic flavonoids. Under the condition, thevetiaflavone had t_R of approx. 16.5 min as in Figure 5B.

methylation products of caffeic acid, 5-hydroxyferulic acid, caffeoyl CoA, 5-hydroxyferuloyl CoA, caffealdehyde, 5-hydroxyconiferylaldehyde, caffeyl alcohol, and 5-hydroxyconiferyl alcohol. On the other hand, the flavonoid apigenin was converted efficiently to its monomethyl analog (Table 2). This product [(I) in Figure 9B] was identified as acacetin, but not the regioisomers genkwanin and thevetiaflavone by comparing the mass spectrum (Figure 9A) and t_R in

the LC chromatogram (Figure 9B) with those of the regioisomers (Figure 9A, B).

The recombinant CtOMT3 did not show OMT activity for matairesinol, while it catalyzed methylation of the flavonoid apigenin to give rise to acacetin efficiently (data not shown).

Kinetic properties of recombinant CtMROMT

The pH optimum for CtMROMT-catalyzed reactions was 7.5, and the kinetic properties of the reaction were characterized at this pH. Lineweaver–Burk analysis revealed that the K_m values for SAM and (\pm)-matairesinols were 3.7 and 9.4 μ M, respectively. The k_{cat} and k_{cat}/K_m values for (\pm)-matairesinols were 0.075 min^{-1} and 8.0 $\text{nM}^{-1} \text{min}^{-1}$, respectively.

Discussion

The recombinant CtMROMT (CtOMT2) catalyzed the regioselective methylation of matairesinol giving rise to arctigenin. The regioselectivity was the same as that of the plant protein. There was no evidence that subsequent methylation of arctigenin occurred to yield 4,4'-O,O-dimethylmatairesinol. On the other hand, CtOMT3 did not methylate matairesinol. An equimolar mixture of recombinant CtMROMT and recombinant CtOMT3 did not affect the matairesinol methylation activity. Instead, the activity was proportional to the content of CtMROMT, suggesting that CtOMT3, when present together with CtMROMT (CtOMT2) in plants, is not an OMT for matairesinol methylation (data not shown). In addition, the expression of *CtMROMT* coincided with the CtMROMT activity profile and arctigenin accumulation in the plant. In contrast, the expression of *CtOMT3* reached the maximum at 12 DAF (Figure 4D) and retained at a high level until 18 DAF, the period when flavonoid accumulation was active (Figure 4C). Taken together, these results indicate that *CtMROMT* is responsible for arctigenin formation in *C. tinctorius*, while *CtOMT3* encodes a flavonoid OMT (named CtFOMT), which will be reported elsewhere. The recombinant CtMROMT also methylated apigenin efficiently and regioselectively to yield acacetin (Table 2, Figure 9), suggesting additional roles for CtMROMT in flavonoid methylation.

The deduced molecular weight of CtMROMT was 39 kDa, and its catalytic activity was independent of Mg^{2+} , showing the typical characteristics of Pl-OMT II [Joshi and Chiang 1998] [or type 1 OMT (Noel et al. 2003)]. This group consists of higher molecular weight OMTs of approximately 38 to 43 kDa that do not require Mg^{2+} for their catalytic activity, while Pl-OMT I [Joshi and Chiang 1998] [or type 2 OMT (Noel et al. 2003)] comprises OMTs with low molecular weight (23 to 27 kDa) and Mg^{2+} -dependent catalytic

activity. CtMROMT showed good amino acid sequence identity with some of the Pl-OMT II members (Figures 6, 7), whereas these OMTs showed diverse substrate specificities. For example, RcOMT3 catalyzed methylation of caffeic acid (Wu et al. 2003), and PtaeAEOMT methylated caffeic acid, 5-hydroxyferulic acid, caffeoyl CoA, and 5-hydroxyferuloyl CoA (Li et al. 1997). VvOMT1 and VvOMT2 showed the greatest activity towards the flavonoid quercetin. In addition, they showed weak activities towards caffeic acid and hydroxypyrazines (Dunlevy et al. 2010). HlOMT1 and HlOMT2 were active towards a number of flavonoid compounds, but only slightly active towards caffeic acid (Nagel et al. 2008). On the other hand, PsOMT1, an (*R,S*)-reticuline 7-*O*-methyltransferase, methylated a number of isoquinoline alkaloids including reticuline as well as guaiaicol, but it did not methylate caffeic acid (Ounarooun et al. 2003). Thus, the identification of CtMROMT extended the diversity of the substrate specificities of the clade composed of Pl-OMT II members (Figure 7).

F. intermedia cell-free extracts catalyzed the methylation of matairesinol to give both arctigenin and isoarctigenin (Ozawa et al. 1993). Because isoarctigenin was not detected in *F. intermedia*, it was proposed that selective formation of the arctigenin moiety of arctiin in the plant could be ascribed to selective glycosylation of matairesinol giving rise to matairesinoside (4-*O*-glucoside of matairesinol) (Figure 2) followed by methylation by the non-regioselective OMT yielding arctiin (Ozawa et al. 1993). These results indicate that CtMROMT probably differs from FiMROMT in its regioselectivity. Further regioselective experiments with purified FiMROMT should be conducted to confirm this assumption.

The present isolation of MROMT will contribute to further studies on the physiological and chemoeological roles of arctigenin and related lignans, in, for example, heartwood formation in conifers (Chang et al. 1999; Chang et al. 2000) and in plant seed germination (Higashinakasu et al. 2005). In addition, all of the genes involved in arctigenin biosynthesis from coniferyl alcohol, that is, those encoding dirigent protein (Gang et al. 1999; Naoumkina et al. 2010; Suzuki and Umezawa 2007; Umezawa 2003a), pinoresinol/lariciresinol reductase (Dinkova-Kostova et al. 1996; Nakatsubo et al. 2008; Naoumkina et al. 2010; Suzuki and Umezawa 2007; Umezawa 2003a), secoisolariciresinol dehydrogenase (Naoumkina et al. 2010; Suzuki and Umezawa 2007; Umezawa 2003a; Xia et al. 2001) and MROMT, are now available. Therefore, it is now possible to produce the antitumor compound arctigenin by synthetic biological strategies in transgenic microorganisms engineered to express arctigenin biosynthetic genes.

The biosynthetic pathways from yatein to another

antitumor compound, podophyllotoxin, were reported by Dewick and co-workers in the 1980s (Dewick 1989) (Figure 1). Later, the pathway from matairesinol to yatein in *Anthriscus sylvestris* (cow parsley) was established by Sakakibara et al. This pathway involves dual methylation (Figure 1): thujaplicatin to 5-*O*-methylthujaplicatin, and 5-*O*-methylthujaplicatin to 4,5-*O,O*-dimethylthujaplicatin (Sakakibara et al. 2003). To give a more complete view of this podophyllotoxin pathway, we recently used CtMROMT as a probe and isolated cDNAs encoding *Anthriscus* lignan OMTs that methylate matairesinol and thujaplicatin. We will report these results in a forthcoming publication.

Conclusions

We report the first molecular cloning of a cDNA encoding a lignan OMT, CtMROMT, which is involved in lignan methylation. The gene encoding this enzyme can be used to study mechanisms of heartwood formation and stimulatory allelopathy during the seed germination stage, and can be applied to the biotechnological production of the antitumor lignans arctigenin and podophyllotoxin.

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