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Original Paper

Substrate-enantiomer selectivity of matairesinol *O*-methyltransferases

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Abstract Previously we reported a cDNA encoding an *O*-methyltransferase (OMT) responsible for the *O*-methylation of matairesinol to afford arctigenin in *Carthamus tinctorius*. However, the regioselectivity and stereoselectivity of its reaction are not yet well understood. In this paper, we report the characterization and comparison of three matairesinol OMTs (MROMTs) encoded by cDNAs isolated from *C. tinctorius* (*CtMROMT*), *Anthriscus sylvestris* (*AsMROMT*), and *Forsythia koreana* (*FkMROMT*). Although they shared matairesinol as a substrate, each recombinant MROMT showed different catalytic behavior. AsMROMT and CtMROMT methylated matairesinol's hydroxyl group at the C4′ position giving rise to arctigenin, while FkMROMT methylated the C4 position hydroxyl group giving rise to isoarctigenin. Analysis of the enantiomeric composition of products from racemic matairesinol showed all OMT reactions to be highly selective in terms of the substrate enantiomers and only use the (−)-enantiomer as the substrate.

Key words: lignan, *O*-methyltransferase (OMT), *Carthamus tinctorius*, *Anthriscus sylvestris*, *Forsythia koreana*.

Lignans are a group of plant secondary metabolites that constitute an abundant class of phenylpropanoids (Koulman et al. 2001; Umezawa 2003) and that have received much interest because of various useful characteristics. For example, many lignans have various biological activities, such as antitumor, antimitotic, and antiviral activities as well as antagonism towards platelet-activating factor and inhibitory activities towards certain enzymes (Harmatha and Dinan 2003; MacRae and Towers 1984; Umezawa 2003; Umezawa et al. 1994). In addition, biosynthetic reactions of lignans involve unique stereochemical properties of great interest in terms of bioorganic chemistry and are expected to provide a model for biomimetic chemistry and its application (Suzuki and Umezawa 2007; Umezawa 2003). Lignans can be classified into three categories depending on the oxidation state of the C9(C9′) positions, located at the terminal of the propyl side chain: lignans with 9(9′)-oxygen, lignans without 9(9′)-oxygen, and dicarboxylic acid lignans (Suzuki and Umezawa 2007; Umezawa 2003). Of the three lignan categories, the study of the biosynthesis of lignans with 9(9′)-oxygen, mostly biosynthesized from coniferyl alcohol, is the most advanced (Suzuki and Umezawa 2007; Umezawa 2003). cDNAs encoding enzymes or proteins that mediate each reaction step in biosynthesis from coniferyl alcohol to matairesinol have been isolated and their recombinant enzymes and proteins characterized in detail. In particular, these studies have demonstrated that optically pure lignans are formed with the aid of dirigent proteins (Davin et al. 1997; Finefield et al. 2012; Halls and Lewis 2002; Pickel et al. 2010; Suzuki and Umezawa 2007; Umezawa 2003) and enzymes involved in post-coupling modification reaction steps after pinoresinol formation (Hemmati et al. 2010; Nakatsubo et al. 2008; Umezawa et al. 2011). Enantiomeric control by dirigent protein is not strong enough to produce optically pure pinoresinol, whereas differential expression of pinoresinol reductase isoforms with distinct selectivities for substrate enantiomers can determine enantiomeric compositions of the product lariciresinol (Nakatsubo et al. 2008).

The pathway from coniferyl alcohol to matairesinol

DDBJ accession numbers for *AsMROMT*, *FkMROMT*, and *CtMROMT* cDNA sequences are AB820127, AB857335, and AB741899, respectively.
Abbreviations: HPLC, high-performance liquid chromatography; LC-MS, Liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; MAFFT, multiple alignment using fast Fourier transform; OMT, *O*-methyltransferase; PCR, polymerase chain reaction
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probably occurs in various plant species and is regarded as the general pathway for lignans with 9(9′)-oxygen, and many lignans originate from lignans on the general pathway. In addition to the enzymes and proteins involved in the general pathway from coniferyl alcohol to matairesinol, cDNAs encoding enzymes that convert lignans of the pathway to various other lignans have been isolated, and their recombinant enzymes characterized; piperitol/sesamin synthase that converts pinoresinol to sesamin via piperitol (Ono et al. 2006), pluviatolide synthase that converts matairesinol to pluviatolide (Marques et al. 2013), and lignan *O*-methyltransferases (OMTs) that methylate matairesinol and thujaplicatin regioselectively to yield arctigenin (Umezawa et al. 2013) and 5-*O*-methylthujaplicatin (Ragamustari et al. 2013), respectively.

However, enantiomeric control of these enzymatic reactions has not been reported. Enantiomeric compositions of dibenzylbutyrolactone lignans, such as matairesinol isolated from various plants, have been determined by chiral high-performance liquid chromatography (HPLC) and all analyzed thus far are found to be optically pure. Hence, enzymes for *in planta* conversion of dibenzylbutyrolaconte lignans might not need stringent enantiomeric controls. In fact, a crude enzyme preparation from *Forsythia intermedia* catalyzed matairesinol methylation to produce both enantiomers of matairesinol monomethyl ethers, namely arctigenin (4′-*O*-methylmatairesinol) and isoarctigenin (4-*O*-methylmatairesinol), although only optically pure arctigenin has been isolated from the plant (Ozawa et al. 1993). Elucidating whether the enzymatic conversion of dibenzylbutyrolactone lignans is enantiomer-selective is not only intriguing in terms of stereoorganic chemistry of natural product biosynthesis but also important for *in vitro* production of optically pure biologically active and useful lignan congeners from racemic mixtures of dibenzylbutyrolactone lignans. This importance is justified by instances in which different enantiomers of lignans possess different biological activities. For example, a levorotatory dibenzylbutyrolactone lignan, (−)-*trans*-methylpluviatolide, has trypanocidal activity, whereas its antipode is inactive (da Silva et al. 2008). Another levorotatory dibenzylbutyrolactone lignan, (−)-matairesinol, and its (+)-enantiomer show different antibacterial activities against *Bacillus subtilis*, *Listeria denitrificans*, and *Staphylococcus aureus* ssp. *Aureus* (Akiyama et al. 2007).

Arctigenin is known to have high pharmacological significance, such as its selective toxicity against cancer cells under glucose starvation (Awale et al. 2006; Kim et al. 2010; Sun et al. 2011), as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase (Eich et al. 1996), for having immunomodulatory effects on tumor necrosis factor-α, nitric oxide production, and lymphocyte production (Cho et al. 1999), and as an activator of AMP-activated protein kinase, which controls whole-body glucose homeostasis (Huang et al. 2012). Even though enantiomeric effects on these activities are not known, it is possible that the activities are affected by the lignans' enantiomeric compositions.

Recently, we have reported the isolation of a cDNA encoding a lignan OMT from *Carthamus tinctorius* (safflower) and characterized its recombinant OMT (Umezawa et al. 2013). The recombinant OMT catalyzed the regioselective *O*-methylation of matairesinol to form arctigenin and was thus designated as *C. tinctorius* matairesinol OMT (CtMROMT). In addition, we also reported a cDNA-encoding OMT (referred to as *AsOMT50*), isolated from *Anthriscus sylvestris*, which encoded a protein with high sequence homology with CtMROMT and also methylated matairesinol (Ragamustari et al. 2013). In this study, another cDNA encoding a matairesinol-methylating OMT (MROMT) was isolated from *Forsythia koreana* and the three recombinant MROMTs were characterized in terms of their selectivity for substrate enantiomers.

Materials and methods

Plant material

A. sylvestris whole plants were collected in April 2006, 2011, and 2012 at the Ashiu Forest Research Station, Field Science Education and Research Center, Kyoto University, Nantan, Japan. The plants were maintained in the experimental garden of Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Uji, Japan. *F. koreana* leaves and flowers were collected in spring 2009 from plants grown in the greenhouse of Suntory Global Innovation Center Ltd.'s Research Department. *C. tinctorius* cv. Round-leaved White developing seeds were collected from plants grown in the RISH experimental garden, as described previously (Umezawa et al. 2013).

Instrumentation

Nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL Ltd., Akishima, Japan).

Gas chromatography-mass spectrometry (GC-MS) was performed with a Shimadzu QP-2010 plus GC-MS system (Shimadzu Corp., Kyoto, Japan), as described previously (Ragamustari et al. 2013).

Reversed-phase HPLC and reversed-phase high-performance liquid chromatography-mass spectrometry (LC-MS) for flavonoid analysis were performed as previously reported (Umezawa et al. 2013).

For purification of matairesinol assay products, a reversed-phase HPLC system composed of a Shimadzu LC-20AD liquid chromatograph equipped with a Shimadzu SPD-20 A UV-Vis detector (Shimadzu Corp.) with detection at 280 nm was used. An XBridge BEH C18 Column (130 Å, 5 μm, and

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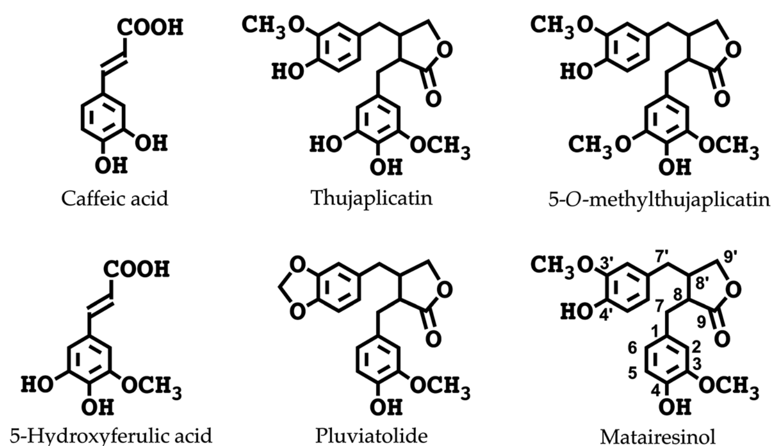

Figure 1. Structure of compounds used as substrates to check substrate specificity and regioselectivity of OMTs.

4.6 mm×150 mm i.d.; Waters Corp., Milford, MA, USA) was used to separate reaction products and substrates at a 0.5 ml min^{-1} flow-rate and a linear gradient solvent system composed of acetonitrile/1% acetic acid in water at t=0 min (20/80 by vol) to t=20 min (40/60) and then held at the latter composition for an additional 20 min.

The optical rotation for arctigenin and isoarctigenin enantiomers was determined using a chiral HPLC system composed of the Shimadzu liquid chromatograph and UV-Vis detector at 280 nm, as described above, and a JASCO OR-990 chiral detector (Jasco Corp., Ltd., Tokyo, Japan). The settings for the chiral detector were polarity at + and gain at ×10, and the chiral column employed was a Chiralpak IB column (4.6 mm×250 mm, 5 μm; Daicel Corp., Osaka, Japan) eluted with an isocratic mobile phase containing ethanol/0.2% acetic acid in *n*-hexane (10/90 by vol) at a flow-rate of 1 ml min^{-1}.

Chiral LC-MS analysis of purified enzyme assay products and arctigenin isolated from *C. tinctorius* developing seeds was performed using an LC-MS system, which consisted of a Shimadzu LC-10AD*vp* HPLC series liquid chromatograph equipped with a Shimadzu SPD-10A*vp* UV-Vis Detector (λ 280 nm) and a Shimadzu LC-MS-2010 A single quadrupole mass spectrometer (Shimadzu Corp.). The system was equipped with an atmospheric pressure chemical ionization interface in positive ion mode. The column used was a Chiralpak IB-3 (2 mm×250 mm, 3 μm; Daicel Corp.) eluted by ethanol/0.2% acetic acid in *n*-hexane (10/90 by vol) at a flow-rate of 0.3 ml min^{-1}. MS parameters were set as previously described, or with a slight modification with the probe voltage at −3.5 kV. The molecular ion $[M+H]^+$ of arctigenin and isoarctigenin at *m*/*z* 373 and of [3-OC^2H_3]arctigenin and [3-OC^2H_3]isoarctigenin at *m*/*z* 376 were monitored.

Chemicals

S-adenosyl-L-methionine (SAM) was purchased from Sigma-Aldrich Japan (Tokyo, Japan); caffeic acid from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); and (±)-matairesinols, (±)-[3-OC^2H_3]matairesinols, (±)-arctigenins, (±)-[3-OC^2H_3]-arctigenins, (−)-arctigenin, (±)-isoarctigenins, (±)-thujaplicatins, (±)-5-*O*-methylthujaplicatins, (±)-pluviatolides, and 5-hydroxyferulic acid were prepared previously (Figure 1) (Nakatsubo et al. 2007; Sakakibara et al. 2003; Sakakibara et al. 2007; Suzuki et al. 2002; Umezawa et al. 2013).

Isolation of arctigenin from C. tinctorius *developing seeds*

A mixture of *C. tinctorius* cv. Round-leaved White developing seeds (14 and 18 day after flowering) was ground in liquid nitrogen and extracted with hot methanol (65°C, 5 ml, and three times). Lipid was removed by hexane extraction and the methanol extract was treated with 58.8 units of β-glucosidase (from almonds, Sigma-Aldrich Japan) in 6.7 ml of 0.1 M sodium acetate buffer at pH 5.0 at 37°C for 24 h. The reaction mixture was then extracted three times with ethyl acetate (7 ml), the extracts taken to dryness, and the residue purified by silica gel chromatography to yield pure arctigenin. The isolated arctigenin was subjected to NMR and GC-MS analysis and the enantiomeric composition determined by chiral LC-MS.

Isolation of OMT *cDNAs*

The *A. sylvestris OMT* cDNA (*AsOMT50*) had been previously isolated (Ragamustari et al. 2013), while the *F. koreana OMT* cDNA (*FkOMT72*) was isolated from a cDNA library made from *F. koreana* leaves and flowers prepared using a ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack3 Gold Cloning Kit (Stratagene Corp., La Jolla, CA, USA), according to the manufacturer's protocol. The resulting *F. koreana* cDNA library was screened according to previously described methods (Ono et al. 2006; Ragamustari et al. 2013), using a probe made from the partial ORF of *CtMROMT* (Umezawa et al. 2013). For RNA-Seq analysis of *F. koreana*, we constructed cDNA libraries and sequenced cDNA by Illumina's next-generation sequencing instrument HiSeq 1000. Total RNAs were extracted from *F. koreana* leaves and shoots using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and were processed according to the TruSeq RNA Sample Prep Kit v2 protocol (Illumina, San Diego, CA, USA). The two cDNA libraries were sequenced using a Hiseq 1000 sequencer

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(Illumina, San Diego, CA, USA) with 100 bp paired-end (PE) reads. The Illumina reads were assembled using CLC Genomics Workbench version 4.8 (CLC Bio, Tokyo, Japan) to obtain assembled contigs (FASTA file, data not shown).

Phylogenetic and sequence analysis

A phylogenetic analysis of the AsOMT50, FkOMT72 and CtMROMT amino acid sequences was conducted using MAFFT (Katoh et al. 2009), and a phylogenetic tree was generated using the neighbor-joining method. *In silico* physical analysis (deduced molecular mass and isoelectric point) was conducted using the *compute pI/Mw* suite available on the ExPASy Server (Gasteiger et al. 2005). The amino acid alignment was visualized using Bioedit (Hall 1999) and the phylogenetic tree viewed and edited using Dendroscope (Huson et al. 2007).

Preparation of recombinant AsOMT50 and FkOMT72

Restriction sites were introduced to the 5′ and 3′ ends of *AsOMT50* and *FkOMT72* ORFs using the gene-specific primers shown in Table 1. After amplification, the PCR products were digested by the corresponding restriction enzymes (New England Biolabs, Inc., Ipswich, MA, USA; Table 1) and ligated into pET23a vectors (Novagen, Inc, Madison, WI, USA; *AsOMT50*) or pCOLD vectors (Takara Bio, Inc., Otsu, Japan; *FkOMT72*) using a T4 ligase kit (Life Technologies, Inc., Carlsbad, CA, USA), according to the manufacturer's protocol. Each construct was then transformed into *E. coli* TOP10 or DH5α competent cells to be amplified and the resulting constructs extracted by NucleoSpin Plasmid QuickPure (Machery-Nagel, Düren, Germany) or QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturers' protocols.

After sequencing to confirm the *OMT* constructs' accuracy, each construct was transformed into *E. coli* BL21 (DE3) competent cells for induction and expression. The yield and folding of recombinant AsOMT50 and FkOMT72 were improved by coexpression with chaperone expressing plasmids, pGro7 and pG-KJE8 (groES-groEL and dnaK-dnaJ-grpE-groES-groEL; Takara Bio, Inc.), respectively. Recombinant protein induction was accomplished by IPTG addition to 1 mM at the logarithmic growth phase of the *E. coli* cultures (OD_{600} at 0.6–1). Induction temperatures were set at 15°C with an incubation time of 24 h. Cells were then collected by centrifugation (2,000×*g*, 4°C, and 10 min) and the resulting pellets stored at −80°C until use.

Substrate specificity of recombinant OMTs

The activity of the recombinant enzymes for potential substrates was checked using *E. coli* cell lysates, which were obtained by sonication of cells from the expression cultures with a Branson Sonifier 250 (Branson Ultrasonic Corp., Danbury, CT, USA; duty cycle, 50 and output control 2 and 3 min) or a Misonix Sonicator S-4000 (Misonix Inc., Farmingdale, NY, USA; 20% amplitude and 3 min). The resulting suspensions were then centrifuged (11,000×*g*, 4°C, and 10 min) and the supernatants used directly in enzyme assays with potential substrates (caffeic acid, 5-hydroxyferulic acid, thujaplicatin, 5-*O*-methylthujaplicatin, matairesinol, or pluviatolide; Figure 1). All assays were conducted in 50 mM Tris-HCl (pH 7.5) buffer, each containing 2 mM $MgCl_2$, 40 μM SAM, 100 μl protein lysate, and 10 μM substrate. Then, 200 μl volumes of the reaction mixtures were incubated at 30°C for 1 h. When caffeic or 5-hydroxyferulic acids were used as the substrate, the reactions were stopped by the addition of 200 μl of 2 N HCl and then extracted with 500 μl of ethyl acetate. For other substrates, the reactions were stopped by extraction with 500 μl of ethyl acetate. The resulting extracts were dried and processed for GC-MS analysis.

Enzyme kinetics of recombinant AsOMT50 and FkOMT72

Kinetic analyses were performed using recombinant OMTs that were purified to homogeneity. For purification, *E. coli* cell pellets from expression cultures were processed and subjected to column chromatography using His-Bind Resin (Novagen, Inc.) according to the manufacturer's protocol with modifications. Modifications were the addition of 10% glycerol and 5 mM ATP in the wash and elution buffers for AsOMT50 recombinant enzymes. These proteins were eluted using buffers containing imidazole at 1 or 0.25 M for AsOMT50 or FkOMT72, respectively. The homogeneity of the purified recombinant proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis using standard methods.

The optimum pH for recombinant OMT assays was determined using potassium phosphate and Tris-HCl buffers at various pH values ranging from 6 to 8.5. Determinations of K_m values for matairesinol were conducted in assays containing 50 mM buffer. Each reaction mixture contained 0.5–5 μM substrate {(±)-matairesinols or (±)-[3-OC^2H_3]-matairesinols}, 40 μM SAM, and 1.58–18.26 μg of recombinant protein. Each reaction mixture (200 μl total vol) was incubated at 30°C for 60 min. Reactions were stopped by extraction with 500 μl of ethyl acetate containing 1 nmol of an internal standard: (±)-[3-OC^2H_3]arctigenins for matairesinol substrate, and (±)-arctigenins or (±)-isoarctigenins for [3-OC^2H_3]-

Table 1. Primers for cloning *OMT*s to expression vectors

OMT	Forward primers	Reverse primers	Restriction sites
AsOMT50	5′-ATAGGATCCATGAGCACATTTATAGGAGAA-3′	5′-ATACTCGAGATCTGA ATAGACTTCGATGAT-3′	*Bam*HI, *Xho*I
FkOMT72	5′-AATAGAGCTCATGGATTCATCCCAAAAGCA-3′	5′-GGCCGAATTCGTTCTT GTACAACTCAATAA-3′	*Sac*I, *Eco*RI

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matairesinol substrate. The reaction products were dried and processed for GC-MS analysis. K_m and V_{max} values were determined by nonlinear regression of Michaelis–Menten plots performed in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Recombinant AsOMT50 and FkOMT72 activity towards the flavonoid apigenin

AsOMT50 and FkOMT50 activities towards the flavonoid apigenin were examined as shown above for CtMROMT (Umezawa et al. 2013), with 10 nmol of apigenin as the substrate instead of matairesinol and incubated at 30°C for 120 min. Dried enzyme assay products were then processed for reversed-phase LC-MS analysis.

Enantiomeric selectivity of AsOMT50, CtMROMT, and FkOMT72-catalyzed reactions

The enantiomeric compositions of products from enzyme assays were determined as described above in the enzyme kinetics section but scaled up proportionately by 10 to 30 times and incubated at 30°C for 240 min. Arctigenin (AsOMT50 and CtMROMT) and [3-OC^2H_3]isoarctigenin (FkOMT72) fractions were collected by reversed-phase HPLC, dried, and processed for chiral LC-MS analysis.

Results

Enantiomeric compositions of lignans

Previously, we reported the enantiomeric compositions of *F. koreana* and *Arctium lappa* lignans, and all dibenzylbutyrolactone lignans obtained were found to be optically pure and levorotatory (Suzuki et al. 2002; Umezawa et al. 1992; Umezawa et al. 1997; Umezawa 2003). The elution order of arctigenin enantiomers using Chiralpak IB columns (Osaka, Japan) are shown in Figure 2. In the present study, chiral LC-MS analysis of arctigenin isolated from *C. tincorius* indicated that this lignan was also optically pure and levorotatory (Figure 7G).

Isolation of cDNAs encoding matairesinol-methylating OMTs and their sequence analysis

Recently, seven plant-*OMT* cDNAs from an *A. sylvestris* cDNA library were isolated in this laboratory and it was noted that one of the recombinant enzymes (AsOMT50) showed matairesinol-methylating activity (Ragamustari et al. 2013). This cDNA had an ORF of 1,062 base pairs (bp) encoding a 353 amino acid protein. Its deduced theoretical isoelectric point and molecular mass were 5.51 and 39.27 kDa, respectively.

In the present study, another plant-*OMT* cDNA was obtained from a *F. koreana* cDNA library and tentatively named *FkOMT72* [DNA Data Bank of Japan (DDBJ) accession number AB857335]. The ORF of *FkOMT72* was 1,086 bp, encoding a 361 amino acid protein. The deduced isoelectric point and molecular mass were 5.66 and 40.05 kDa, respectively. Alignment of AsOMT50, FkOMT72, CtMROMT, and other previously reported plant OMTs showed that all these OMTs possessed conserved motifs of plant OMTs, including catalytic, SAM-binding, and substrate-binding residues, as previously proposed (Figure 3) (Joshi and Chiang 1998; Zubieta et al. 2001).

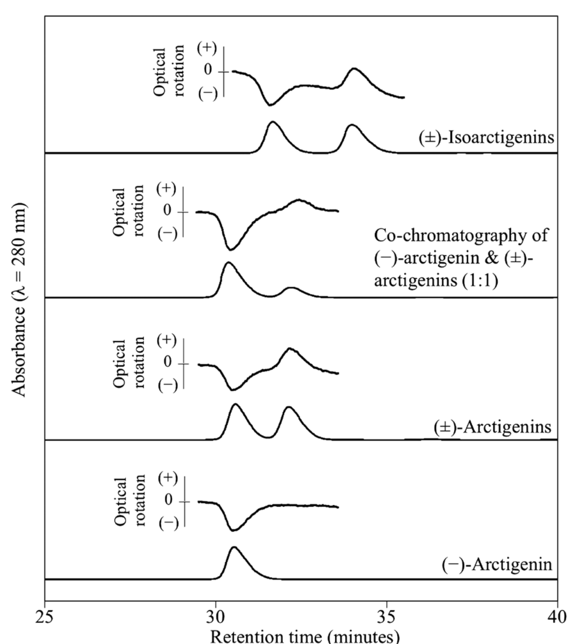

Figure 2. Chiral HPLC of racemic isoarctigenin, arctigenin, and (−)-arctigenin isolated from *A. lappa* (Suzuki et al. 2002).

A phylogenetic tree consisting of AsOMT50, FkOMT72, and previously reported plant OMTs is shown in Figure 4. AsOMT50 had the highest amino acid sequence identity with CtMROMT (Umezawa et al. 2013) (85.5%). On the other hand, FkOMT72 was most closely related in the phylogenetic tree to AsTJOMT (Ragamustari et al. 2013) (amino acid sequence identity of 52.8%).

To explore additional FkOMTs, we conducted RNA-Seq analysis of *F. koreana* by next generation sequencer (NGS). We subsequently screened *FkOMT* cDNAs showing structural similarity with *CtMROMT* and *AsMROMT* from RNA-Seq data *in silico*, and found at least four putative *FkOMT* cDNAs. Interestingly, no striking homolog for *CtMROMT* and *AsMROMT* was found (the highest amino acid identity of the putative FkOMTs with the MROMTs was only 35%).

Substrate specificity and regioselectivity of recombinant AsOMT50 and FkOMT72

AsOMT50 and *FkOMT72* cDNAs were individually expressed in *E. coli* cells. Crude recombinant proteins obtained from these cells were individually submitted

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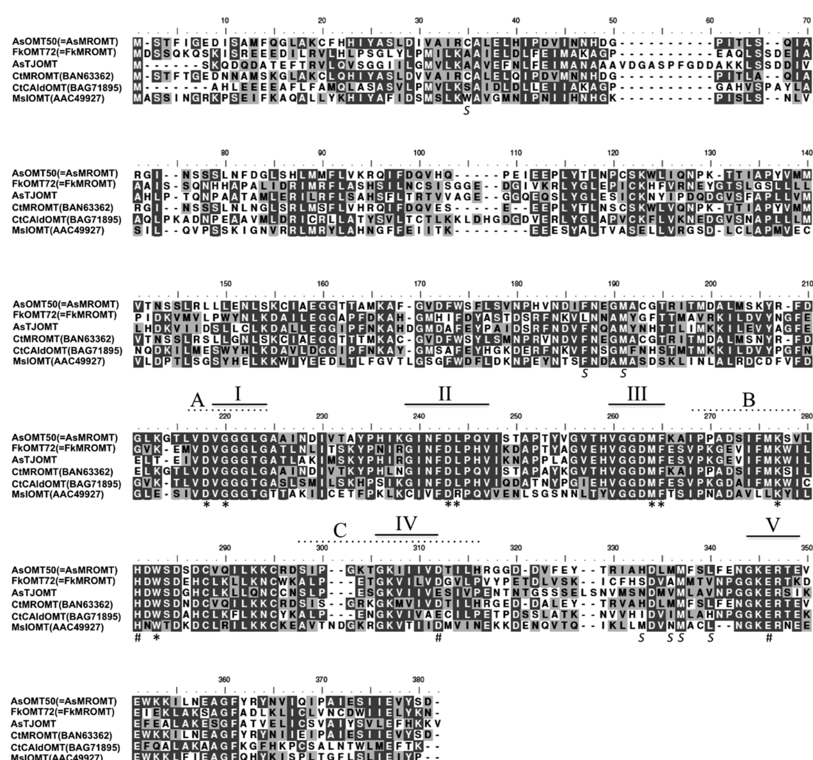

Figure 3. Alignment of the predicted amino acid sequence of AsOMT50 (AsMROMT), FkOMT72 (FkMROMT), and related OMTs. SAM-binding motifs A, B, and C and regions I, II, III, IV, and V are domains conserved among plant OMTs. Catalytic (#), SAM-binding (*), and substrate-binding (*S*) residues were determined from the *Medicago sativa* isoflavone OMT (Zubieta et al. 2001).

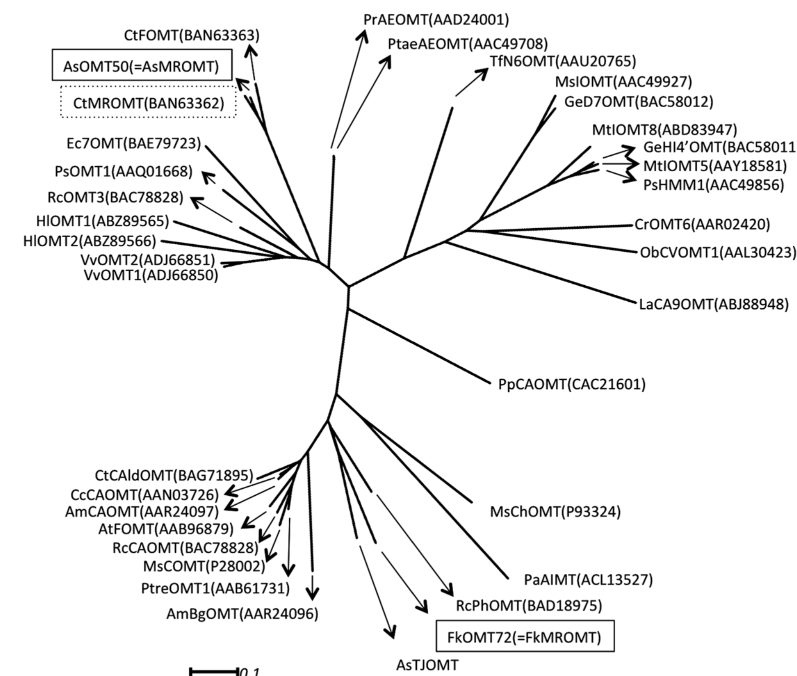

Figure 4. Phylogenetic tree of AsOMT50 (AsMROMT), FkOMT72 (FkMROMT) with related OMTs created by the neighbor-joining method from amino acid alignment generated by MAFFT.

to enzyme assays with potential substrates, including caffeic acid, 5-hydroxyferulic acid, thujaplicatin, 5-*O*-methylthujaplicatin, matairesinol, or pluviatolide (Figure 1). GC-MS analysis of the assay products [trimethylsilyl (TMS) ethers] showed that recombinant AsOMT50 and FkOMT72 only accepted matairesinol as their substrate among the compounds tested. In addition, no evidence was obtained for the formation of dimethylmatairesinol

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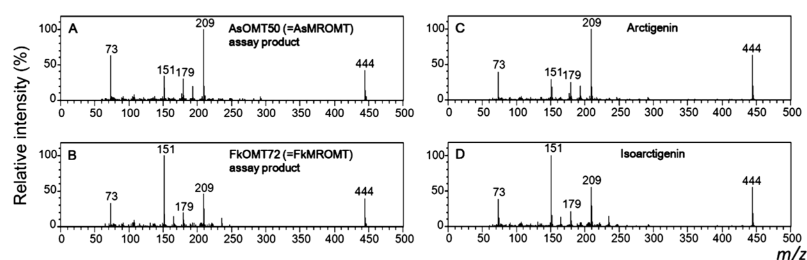

Figure 5. Mass spectrum of AsOMT50 assay product (A) and FkOMT72 assay product (B) after incubation with matairesinol compared with those of authentic arctigenin (C) and isoarctigenin (D). From mass spectrum and retention time comparisons, products of AsOMT50 and FkOMT72-mediated methylation of matairesinol are unequivocally arctigenin and isoarctigenin, respectively.

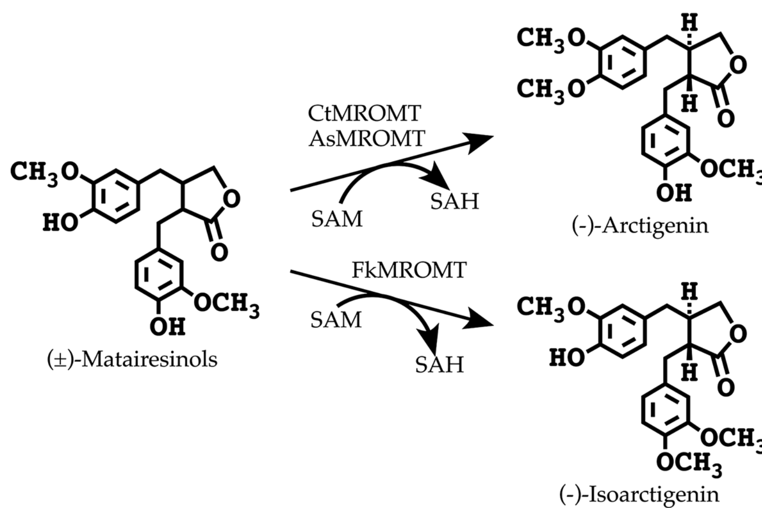

Figure 6. *O*-Methylation scheme catalyzed by AsMROMT, CtMROMT (Umezawa et al. 2013), and FkMROMT.

Table 2. Kinetic constants of AsMROMT and FkMROMT with matairesinol

	K_m (mM)	V_{max} (nmol min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
AsMROMT	$3.1\pm0.66\times10^{-3}$	$2.1\pm0.23\times10^{-3}$	$53.7\pm5.9\times10^{-3}$	16.9
FkMROMT	$4.1\pm1.2\times10^{-3}$	$1.1\pm0.18\times10^{-3}$	$5.0\pm0.84\times10^{-3}$	1.3

by either OMT.

The recombinant OMTs were purified to apparent homogeneity and used individually in OMT assays. GC-MS analysis of the products (TMS ethers) obtained from incubation of (±)-matairesinols with recombinant AsOMT50 had the same mass spectrum (Figure 5A) and retention time (16.05 min) as those of authentic arctigenin (TMS ether, mass spectrum, Figure 5C; retention time, 16.03 min), but not authentic isoarctigenin (TMS ether, mass spectrum, Figure 5D; retention time, 16.46 min). On the other hand, the product with recombinant FkOMT72 showed a mass spectrum and retention time (Figure 5B and 16.46 min, respectively) identical to those of authentic isoarctigenin but not arctigenin.

LC-MS analysis revealed that AsOMT50 and FkOMT72 assays with the flavonoid apigenin did not give rise to any product (data not shown).

Thus, regardless of the product isomers, both AsOMT50 and FkOMT72 catalyzed matairesinol monomethylation and thereby AsOMT50 and FkOMT72 were re-designated and hence are referred to as *A. sylvestris* matairesinol OMT (AsMROMT) and *F. koreana* matairesinol OMT (FkMROMT), respectively (Figure 6).

Kinetic analysis of AsMROMT and FkMROMT

GC-MS analyses of AsMROMT and FkMROMT assay products were performed to determine K_m, V_{max}, and k_{cat} values for AsMROMT and FkMROMT towards matairesinol (Table 2). Michaelis–Menten nonlinear regression analyses revealed that the K_m and V_{max} values for AsMROMT were $3.1\pm0.66\times10^{-3}$ mM and $2.1\pm0.23\times10^{-3}$ nmol·min^{-1}, respectively, when 1.58 μg of recombinant enzyme was used in the assay. Assuming AsMROMT was a monomer, the E_t value was calculated by dividing the recombinant AsMROMT amount in the reaction (1.58 μg) by its deduced molecular mass (39,270 Da), resulting in 0.04 nmol. The k_{cat} value

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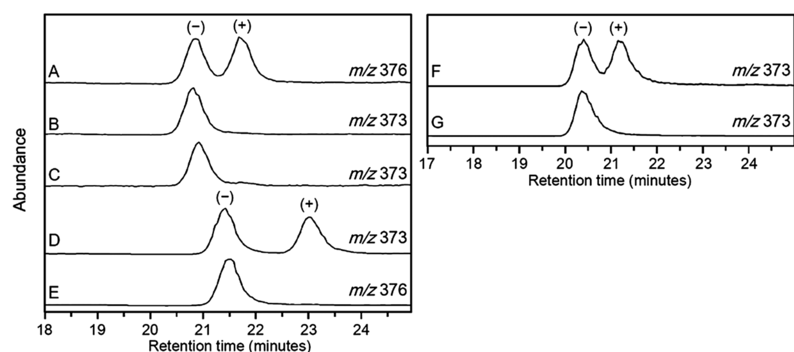

Figure 7. Chiral selected ion monitoring chromatograms of authentic (±)-[3-OC^2H_3]arctigenins (A), enzymatic reaction product from CtMROMT assays incubated with (±)-matairesinols (B), enzymatic reaction product from AsMROMT assays incubated with (±)-matairesinols (C), authentic (±)-isoarctigenins (D), enzymatic reaction product from FkMROMT assays incubated with (±)-[3-OC^2H_3]matairesinols (E), authentic (±)-arctigenins (F), and arctigenin isolated from *C. tinctorius* developing seeds (G). Retention time difference between A–C and F–G was due to separate analyses.

(V_{max}/E_t) for AsMROMT was 53.7±5.9×10^{-3} min^{-1}, and its catalytic efficiency (k_{cat}/K_m) was 16.9 min^{-1} mM^{-1}. K_m and V_{max} values for FkMROMT were 4.1±1.2×10^{-3} mM and 1.1±0.18×10^{-3} nmol·min^{-1}, respectively, with 8.65 μg of recombinant enzyme. Assuming FkMROMT was a monomer, in a similar manner as above, the E_t value, from dividing the recombinant FkMROMT amount (8.65 μg) by its deduced molecular mass (40,052 Da), resulted in 0.21 nmol. The k_{cat} value (V_{max}/E_t) for FkMROMT was 5.0±0.84×10^{-3} min^{-1}, and its catalytic efficiency (k_{cat}/K_m) was 1.3 min^{-1} mM^{-1}. At greater substrate concentrations, we observed substrate inhibition. Thus, elucidating the detailed mechanisms awaits further experiments.

Enantiomeric composition of products from recombinant MROMT assays

Chiral LC-MS analysis showed that following incubation of (±)-matairesinols with AsMROMT or CtMROMT, only (−)-arctigenin was formed, but not (+)-arctigenin (Figure 7A–C). FkMROMT assays with (±)-[3-OC^2H_3]matairesinols also showed selectivity towards the (−)-enantiomer substrate, giving rise only to (−)-[3-OC^2H_3]isoarctigenin (Figure 7D, E).

Discussion

In the present study, first the enantiomeric selectivity of regioselective matairesinol methylation to form arctigenin by *C. tinctorius* MROMT (Figure 6) was examined and LC-MS analysis indicated that the product from racemic (±)-matairesinols was the optically pure (−)-enantiomer (Figure 7B). This was in good accordance with optically pure (−)-arctigenin isolated from the plant species (Figure 7G). In addition, AsMROMT, which shared high amino acid sequence homology with CtMROMT, also catalyzed regioselective matairesinol methylation to yield arctigenin and again the reaction product from (±)-matairesinols, arctigenin, was optically pure (−)-enantiomer (Figure 7C). Arctigenin was detected in *A. sylvestris*, but its content was very low (Koulman et al. 2001). In fact, this lignan was not detected in our plant specimen (data not shown), and the enantiomeric composition of arctigenin in *A. sylvestris* could not be determined.

These results clearly indicated that MROMTs for the conversion of matairesinol to arctigenin were highly selective in terms of substrate enantiomers under these conditions. As such, OMTs could be exploited for the *in vitro* production of optically pure arctigenin and for the production of optically pure residual (+)-matairesinol from racemic (±)-matairesinols. It was also worth noting that although sharing high amino acid sequence identity as well as the same enantiomer and regioisomer selectivities in matairesinol methylation, CtMROMT and AsMROMT showed differences in flavonoid methylation. CtMROMT efficiently methylated the flavonoid apigenin to its methyl ether derivative, acacetin, whereas AsMROMT was not able to accept apigenin as a substrate.

The strictness of these MROMTs in terms of both enantiomer and regioisomer selectivities was in sharp contrast to the case of methylation by MROMT activity in *F. intermedia* crude enzyme preparation. Following incubation of (±)-matairesinols with the crude enzyme, both enantiomers of two regioisomers, arctigenin and isoarctigenin, were formed, although the plant produces only optically pure (−)-arctigenin but not isoarctigenin (Ozawa et al. 1993). Similar to *F. intermedia*, *F. koreana* produced optically pure (−)-matairesinol and (−)-arctigenin (Umezawa et al. 1992). To isolate a cDNA encoding *Forsythia* MROMT, we screened a cDNA library prepared from *F. koreana*, and obtained a cDNA encoding a putative OMT, which was tentatively named *FkOMT72*.

FkOMT72 showed rather low amino acid sequence

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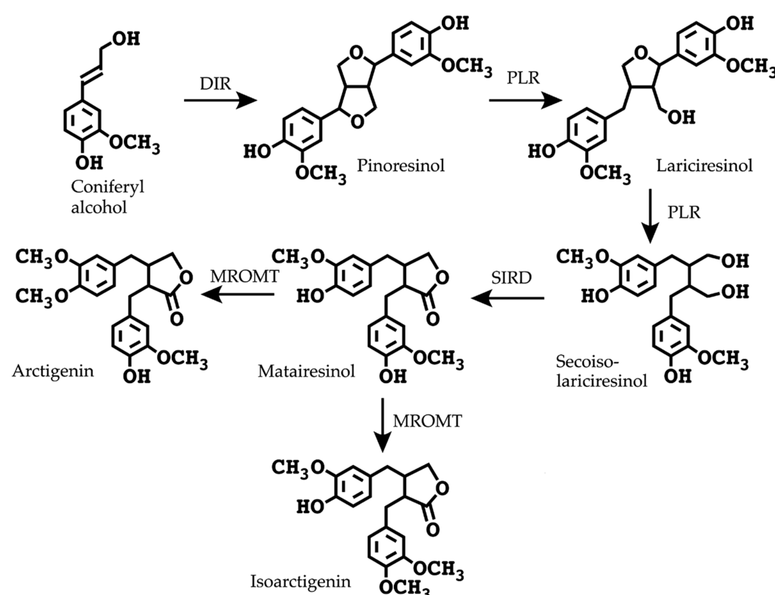

Figure 8. The lignan biosynthetic pathway from coniferyl alcohol to arctigenin via pinoresinol, lariciresinol, secoisolariciresinol, and matairesinol. DIR, dirigent protein; PLR, pinoresinol/lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase; and MROMT, matairesinol OMT.

identity with CtMROMT and AsMROMT (31.9 and 32.5%, respectively), while it shared moderate identity with AsTJOMT (52.8%, Figure 3). Despite the low sequence identity with the former two MROMTs, biochemical characterization of recombinant FkOMT72 showed matairesinol monomethylation activity, and the OMT was redesignated as FkMROMT. However, the methylation product was unequivocally identified as isoarctigenin and not the naturally occurring regioisomer, arctigenin (Figure 5). The formed isoarctigenin was found to be the optically pure (−)-enantiomer (Figure 7E). This MROMT activity probably corresponded with isoarctigenin formation by the MROMT activity of crude *F. intermedia* enzyme (Ozawa et al. 1993). However, the physiological role of FkMROMT awaits further studies. The present result suggested the presence of another *F. koreana* OMT that catalyzes matairesinol methylation to produce arctigenin, similar to CtMROMT and AsMROMT. RNA-seq analysis suggested that FkOMTs responsible for arctigenin formation in *F. koreana* are likely to be phylogenetically distant from CtMROMT and AsMROMT.

Enantiomer-selective OMT activity has also been previously reported in *Berberis* species (Muemmler et al. 1984). An OMT was isolated from *Berberis* cell cultures, which showed selectivity towards the alkaloid (*S*)-scoulerine, but not (*R*)-scoulerine, to afford (*S*)-tetrahydrocolumbamine. Another report has shown that 2-hydroxyisoflavanone 4′-OMT from licorice (*Glycyrrrhiza echinata*) and (+)-6a-hydroxymaackiain 3-*O*-methyltransferase from pea (*Pisum sativum*) exhibit selectivity towards the (+)-enantiomers instead of (−)-enantiomers of their substrates (Akashi et al. 2006).

Understanding the biosynthetic pathways of plant-derived compounds and the selective mechanisms that occur, such as those in the biosynthesis of lignans (Figure 8), can lead to new strategies in improving efficiency for organic syntheses of chiral pharmaceuticals (Marques et al. 2013; Yamamura et al. 2010). To our knowledge there have been no reports to date regarding the strict selectivity for substrate enantiomers by OMTs in lignan biosynthesis. In this study, MROMTs from *C. tinctorius*, *A. sylvestris*, and *F. koreana* were shown to have strict regioselectivity and selectivity in terms of substrate enantiomers.

Conclusions

Research into the characterization of the genes and enzymes related to the lignan biosynthetic pathway is important for increasing efficiency of related chemical production in the pharmaceutical industry. This study reports the characterization of three recombinant lignan OMTs that possess strict regioselectivity and selectivity in terms of substrate enantiomers in catalyzing matairesinol methylation to either (−)-arctigenin or (−)-isoarctigenin. Elucidating the structural basis for these selectivities through protein modelling and site-directed mutagenesis would be an interesting follow-up investigation to this research.

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