*Plant Biotechnology* 30, 375–384 (2013)
DOI: 10.5511/plantbiotechnology.13.0527b

***Original Paper***

# A novel *O*-methyltransferase involved in the first methylation step of yatein biosynthesis from matairesinol in *Anthriscus sylvestris*

Safendrri Komara Ragamustari[1,2], Tomoyuki Nakatsubo[1], Takefumi Hattori[1,3], Eiichiro Ono[4], Yu Kitamura[1], Shiro Suzuki[1], Masaomi Yamamura[1], Toshiaki Umezawa[1,2,*]

[1]Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan; [2]Institute of Sustainability Science, Kyoto University, Uji, Kyoto 611-0011, Japan; [3]Institute of Socio-Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan; [4]Research Department, Suntory Global Innovation Center Ltd., Mishima, Osaka 618-8503, Japan
*E-mail: tumezawa@rish.kyoto-u.ac.jp Tel: +81-774-38-3625 Fax: +81-774-38-3682

Received May 5, 2013; accepted May 27, 2013 (Edited by T. Aoki)

**Abstract** Yatein is an important biosynthetic precursor of the lignan podophyllotoxin. In *Anthriscus sylvestris*, yatein biosynthesis is preceded by two regioselective methylations involving the intermediates: thujaplicatin and 5-*O*-methylthujaplicatin. The two methylation steps are most likely catalyzed by plant *O*-methyltransferases (OMTs). In this paper, we report the isolation and characterization of a cDNA encoding an OMT involved in the first methylation step. The *OMT* cDNA was isolated from an *A. sylvestris* cDNA library prepared from roots and young shoots. The *OMT* was expressed as a recombinant protein using the pET expression system. Of the substrates that were tested, the recombinant OMT exclusively catalyzed regioselective methylation of thujaplicatin to produce 5-*O*-methylthujaplicatin, and thus was designated as *A. sylvestris* thujaplicatin OMT (AsTJOMT). Kinetic analysis with its substrate (thujaplicatin) showed that AsTJOMT had a $K_m$ value of 3.8 $\mu$M and $k_{cat}$ value of 0.29 $min^{-1}$. Quantitative real-time polymerase chain reaction showed that *AsTJOMT* had the highest expression level in roots compared with other organs. This was in accordance with plant protein assays in which specific activity for thujaplicatin was significantly higher in roots compared with other organs. To the best of our knowledge, this is the first report on the isolation and characterization of a thujaplicatin-specific plant OMT.

**Key words:** Lignan, yatein, *O*-methyltransferase (OMT), thujaplicatin, *Anthriscus sylvestris*.

Lignans are phenylpropanoid dimers that are linked by the central carbon (C8) of their propyl side chains, and are known for their important biological characteristics (Umezawa 2003; Suzuki and Umezawa 2007; Nakatsubo et al. 2008b). The lignan yatein is an important typical heartwood lignan and a key biosynthetic intermediate of podophyllotoxin (Sakakibara et al. 2003). Podophyllotoxin is a pharmacologically important compound that has received much attention. It is used as a precursor for the chemical synthesis of the anticancer drugs etoposide, teniposide and etopophos (Farkya et al. 2004). However, sources of podophyllotoxin are becoming increasingly scarce in nature. As a result, podophyllotoxin availability and biosynthesis have become the main focus of many researchers (Farkya et al. 2004; Kadkade 1982; Marques et al. 2013; Seidel et al. 2002).

*Anthriscus sylvestris* is a good source of yatein and deoxypodophyllotoxin, which are precursors of podophyllotoxin (Sakakibara et al. 2003; Van Uden et al. 1997; Umezawa 2003; Suzuki and Umezawa 2007). Furthermore, *A. sylvestris* roots have been used as a raw material to make drugs (Kozawa et al. 1982). The plant itself has been reported to accumulate a significant amount of lignans and other cytotoxic and antiproliferative compounds in its organs (Ikeda et al. 1998; Jeong et al. 2007; Koulman et al. 2003; Lim et al. 1999), which makes it a good experimental plant for studying lignan biosynthesis.

Through feeding experiments, Sakakibara et al. (2003) proposed a biosynthetic pathway for yatein in *A. sylvestris* that included two methylations most likely catalyzed by plant *O*-methyltransferases (OMTs) (Figure 1). These two steps involve the conversion of

Abbreviations: GC-MS, gas chromatography-mass spectrometry; MAFFT, multiple alignment using fast Fourier transform; OMT, *O*-methyltransferase; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR.
This article can be found at http://www.jspcmb.jp/
Published online September 12, 2013

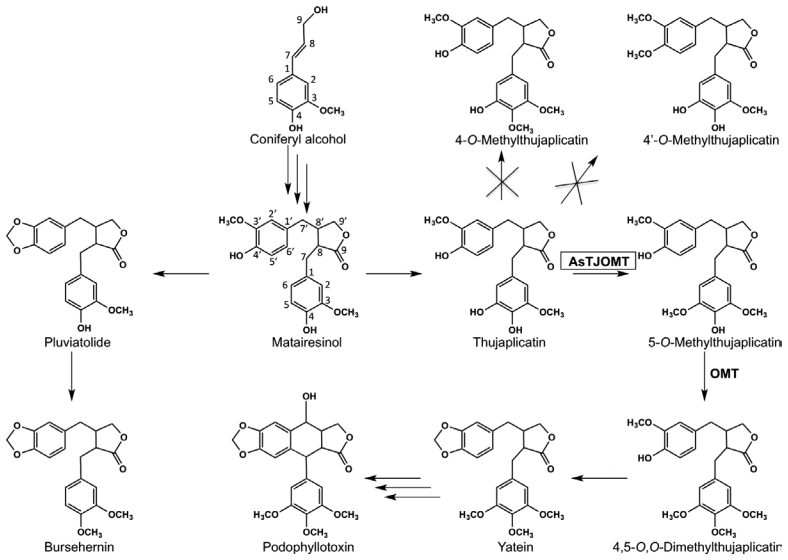


Figure 1. Proposed yatein biosynthesis pathway in *A. sylvestris* based on feeding experiments (Sakakibara et al. 2003).

thujaplicatin to 5-*O*-methylthujaplicatin and 5-*O*-methylthujaplicatin to 4,5-*O*,*O*-dimethylthujaplicatin.

Methylation by plant OMTs involves the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a specific hydroxyl group of the substrate, which gives rise to *S*-adenosyl-L-homocysteine and a methyl ether derivative of the substrate. Plant OMTs are involved in the biosynthesis of natural plant compounds, including lignin, lignans and flavonoids (Ibrahim et al. 1998; Joshi and Chiang 1998; Schröder et al. 2002; Umezawa 2010; Umezawa et al. 2013; Zubieta et al. 2001).

Elucidation of the OMT-encoding and other genes involved in the biosynthetic pathway of podophyllotoxin will enable us to conduct biotechnological manipulation of cell cultures or whole plants for more efficient podophyllotoxin production. Recently, we have reported a cDNA encoding a lignan OMT (*Carthamus tinctorius* matairesinol OMT, CtMROMT) (Umezawa et al. 2013). However, cDNAs encoding enzymes involved in the pathway from matairesinol to yatein shown in Figure 1 have not yet been isolated.

In this research, we isolated and characterized a cDNA encoding an OMT involved in the first methylation step of yatein biosynthesis in *A. sylvestris*.

## Materials and methods

### *Plant material*

*A. sylvestris* plants were collected in April 2006, April 2011, and April 2012 at the Ashiu Forest Research Station, Kyoto University, Ashiu, Kyoto. The plants were maintained in the experimental garden of the Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto. All plant organs used in the experiments were collected in springtime.

### *Instrumentation*

Gas chromatography mass spectrometry (GC-MS) was performed with a QP-2010 plus GC-MS system [electron-impact mode (70 eV)] (Shimadzu Co., Kyoto, Japan); column, HiCap CBP10-M25-025 column (10 m×0.22 mm) (Shimadzu); carrier gas, helium; injection temperature, 250°C; column temperature, 160°C at $t=0$ to 2 min, then raised to 250°C at 10°C min$^{-1}$.

### *Chemicals*

SAM was purchased from Sigma-Aldrich Japan (Tokyo, Japan); caffeic acid (CA) was purchased from Tokyo Chemical Industry (Tokyo, Japan); (±)-matairesinols, (±)-thujaplicatins, (±)-5-*O*-methylthujaplicatins, (±)-4-*O*-methylthujaplicatins, (±)-[3,5-($OC^2H_3$)$_2$]-5-*O*-methylthujaplicatins, (±)-[3,5-($OC^2H_3$)$_2$]-4,5-*O*,*O*-dimethylthujaplicatins, (±)-pluviatolides, and 5-hydroxyferulic acid (5-HFA) were prepared previously (Li et al. 2000; Nakatsubo et al. 2008a; Sakakibara et al. 2003; Sakakibara et al. 2007; Umezawa et al. 2013). (±)-4′-*O*-Methylthujaplicatins were prepared in a similar manner to the synthesis of (±)-4-*O*-methylthujaplicatins [the mass spectrum (TMS ether): Figure 8c].

### *Thujaplicatin OMT (TJOMT)-specific activity of plant proteins extracted from* A. sylvestris *organs*

Using a mortar and pestle, *A. sylvestris* organs (root, stem, leaf, and young shoot; approx. 0.5 g) were pulverized in liquid nitrogen and homogenized on ice for approximately 5 min in 2 ml extraction buffer (0.1 M potassium phosphate, pH 7.5) containing 0.01 M dithiothreitol, 0.1 g polyvinylpolypyrrolidone (polyclar AT), and 0.1 g sea sand. The sample was ground until it became a slurry and then centrifuged at 10,000×*g* at 4°C for 15 min. The supernatant was filtered through a Millex-GV PVDF filter (pore size, 0.22 μm Millipore, Carrigtwohill Co., Cork, Ireland) and applied to a Sephadex G-25 (Pharmacia,

Uppsala, Sweden) column to change the buffer to 50 mM potassium phosphate buffer (pH 7). The obtained protein solution was immediately used for enzymatic assays with 10 $\mu$M of thujaplicatin or 5-*O*-methylthujaplicatin and 0.04 mM of SAM in 50 mM potassium phosphate buffer (pH 7). One hundred microliters of plant protein preparation was added into the enzyme mixture. Control reactions were performed using a boiled plant protein preparation (boiled at 95°C for 5 min) in the enzyme mixture, removing SAM or removing the substrate from the enzyme mixture. We also conducted assays that included 2 mM of $MgCl_2$ to assess the effect of $Mg^{2+}$ ions on OMT catalysis. The reaction was initiated by adding the enzyme preparation, and incubated at 30°C for 60 min. The reaction was terminated by extraction with 0.5 ml of ethyl acetate containing 1 nmol of (±)-[3,5-($OC^2H_3)_2$]-5-*O*-methylthujaplicatin (when thujaplicatin was used as the substrate) or 1 nmol of (±)-[3,5-($OC^2H_3)_2$]-4,5-*O*,*O*-dimethylthujaplicatin (when 5-*O*-methylthujaplicatin was used as the substrate). The solvent was removed from the ethyl acetate solutions under a high vacuum. The reaction products obtained were dissolved in *N*,*O*-bis(trimethylsilyl)acetamide (10 $\mu$l). After standing at 60°C for 45 min, an aliquot (0.8 $\mu$l) of the solution was analyzed by GC-MS using a Shimadzu QP-2010 plus GC-MS system.

### *Total RNA isolation and reverse transcription*

Total RNAs were extracted from organs of *A. sylvestris* using the Plant RNeasy extraction kit (buds and leaves) (Qiagen GmbH, Hilden, Germany) or a modified CTAB protocol (roots and stem) based on the method described by Chang et al. (1993). In the modified CTAB protocol, we omitted L-spermidine and $\beta$-mercaptoethanol from the extraction buffer. An aliquot of the total RNA from each organ was reverse-transcribed using Superscript II reverse transcriptase (Life Technologies Corp., Carlsbad, CA, USA) and random hexamers according to the manufacturer's protocol.

### *Construction of an* A. sylvestris *cDNA library and screening of O*-methyltransferase *cDNAs*

Poly(A)$^+$ RNA was obtained from *A. sylvestris* total RNA isolated from a mixture of root and young shoots. The poly(A)$^+$ RNA was used to construct a cDNA library in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA), which was carried out by Takara Bio Inc. (Otsu, Shiga, Japan).

*OMT* cDNAs were screened from the prepared cDNA library, which had a titer of $4.4\times10^9$ plaque-forming units/ml. To screen the cDNA library, probes from partial open reading frames of four *OMT* cDNAs [*CtMROMT* (Umezawa et al. 2013), *Pinus taeda hydroxycinnamic acids/hydroxycinnamoyl CoA esters OMT* (Li et al. 1997), *Populus tremuloides caffeic acid OMT* (Bugos et al. 1991), *C. tinctorius 5-hydroxyconiferaldehyde OMT* (*CAldOMT*) (Nakatsubo et al. 2007)] were prepared by PCR-based DIG labeling (Roche, Mannheim, Germany). Approximately 50,000 plaque-forming units of the cDNA library were screened with a mixture of the four DIG-labeled plant-*OMT* probes with low stringency. Screening and detection of positive clones were performed with DIG-DNA labeling and detection kit (Roche) as described previously (Ono et al. 2006).

Positive clones were subjected to sequencing with the BigDye-terminator version 3.1 cycle sequencing kit (Applied Biosystems LLC, Foster City, CA, USA), blastx analysis and contig assembly using the CAP program (Huang 1992) [available in the bioedit software package (Hall 1999)]. Seven independent putative *OMT* open reading frames (ORFs) were identified and subjected to phylogenetic analysis using MAFFT (Katoh et al. 2009) and physical analysis (deduced molecular mass and isoelectric point) using the Protein Identification and Analysis Tools on the ExPASy Server (Gasteiger et al. 2005).

### *Expression of recombinant* A. sylvestris *OMTs (AsOMTs) in pET system*

Restriction sites were introduced to the 5′ and 3′ ends of 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., Beverly, MA, USA) (Table 1) and ligated into pET23a vectors (Novagen, San Diego, CA, USA) using a T4 ligase kit (Life Technologies) according to the manufacturer's protocol.

Each construct was then transformed into *E. coli* TOP10 or DH5$\alpha$ competent cells to be amplified. The constructs were extracted with the NucleoSpin Plasmid QuickPure kit (Machery-Nagel, Düren, Germany) according to the manufacturer's protocol. After sequence confirmation, the constructs were transformed into *E. coli* BL21 (DE3) competent cells for induction and expression.

Production of recombinant AsOMTs was done using the Overnight Express Kit I (Novagen) according to the

Table 1. Primers used for *AsOMT* subcloning into pET23a expression vectors.

| *AsOMT* | Forward primer | Reverse primer | Restriction sites |
|---|---|---|---|
| *AsOMT15* | 5′-GGCGAATTCATGCAGATAGTAAATTCTTCC-3′ | 5′-GGTTCTCGAGCATTATCTTGTACAACTCAA-3′ | *Eco*RI, *Xho*I |
| *AsOMT29* | 5′-GGAATTCGCTAGCATGAATACAGACACT-3′ | 5′-ATTTCTCGAGTTTGTAGTATTCGATAATCC-3′ | *Nhe*I, *Xho*I |
| *AsOMT50* | 5′-GGAATTCCATATGATGTTCCAAGGCCTTGC-3′ | 5′-ATTTGCGGCCGCATCTGAATAGACTTCGAT-3′ | *Nde*I, *Not*I |
| *AsOMT58* | 5′-GGCGAGCTCATGAACACAACAACTGAGCTT-3′ | 5′-ACTTGCGGCCGCCTTGAGATATTCGATAAC-3′ | *Sac*I, *Not*I |
| *AsOMT95* | 5′-GGAATTCGCTAGCATGGCAATAGTATCTGC-3′ | 5′-GGTTCTCGAGCATGTTCTTATAGCACTCGA-3′ | *Nhe*I, *Xho*I |
| *AsOMT116* (=*AsTJOMT*) | 5′-GGAATTCGCTAGCATGTCTAAACAAGATC-3′ | 5′-ATTTCTCGAGCACTTTCTTATGAAATTCTA-3′ | *Nhe*I, *Xho*I |
| *AsOMT148* | 5′-GGCGAATTCATGGCGGACATCAAGATCAAG-3′ | 5′-TGCTAGGTTCTCGAGCTTGGAAAATTCCAT-3′ | *Eco*RI, *Xho*I |

manufacturer's protocol. After induction, the cells were pelleted by centrifugation (2,000×*g*, 4°C, 10 min) and stored at −80°C until use.

### *Substrate specificity of recombinant AsOMTs*

To check whether the recombinant enzymes had any activity with potential substrates, pellets from the expression cultures were lysed by sonication using a Branson Sonifier 250 (Branson Ultrasonic Corp., Danbury, CT) (duty cycle: 50, output control: 2). The sonicated pellet suspensions were then centrifuged (11,000×*g*, 4°C, 10 min) and the supernatant was used directly in an enzyme assay with the potential substrates (CA, 5-HFA, thujaplicatin, 5-*O*-methylthujaplicatin, matairesinol, and pluviatolide). All assays were conducted in 50 mM Tris-HCl (pH 7.5) buffer, each containing 2 mM $MgCl_2$, 0.04 mM SAM, 100 $\mu$l protein lysate, and 10 $\mu$M of substrate. Each reaction mixture (200 $\mu$l) was incubated at 30°C for 1 h. When CA and 5-HFA were used as the reaction substrate, the reaction was stopped by the addition of 200 $\mu$l of 2 N HCl, and then extracted with 500 $\mu$l ethyl acetate. The ethyl acetate extracts were dried and subjected to GC-MS analysis.

### *Kinetic properties of recombinant AsOMT116 (AsTJOMT)*

Kinetic analysis was done using recombinant AsOMT116 that was purified to homogeneity. For purification, the cell pellets were processed and subjected to column chromatography using His-Bind Resin (Novagen) according to the manufacturer's protocol. Homogeneity of the purified recombinant protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods.

Determination of the recombinant AsOMT116 $K_m$ value for thujaplicatin was conducted in assays containing 50 mM potassium phosphate buffer (pH 7). Each reaction mixture contained 0.5–5 $\mu$M thujaplicatin, 40 $\mu$M SAM, and 1.21 $\mu$g recombinant protein. $K_m$ and $V_{max}$ values were determined from Lineweaver–Burk plots. Each reaction mixture (200 $\mu$l) was incubated at 30°C for 30 min.

### *Gene expression analysis*

For gene expression analysis of *AsOMT116*, quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Universal Master Mix (Applied Biosystems) with a 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol at default parameters. The PCR primers used were as follows: forward primer, 5′-CAG TAA ATC CGG GAG GAA AGG-3′, reverse primer, 5′-GTT CTA CGG TGG CGA ATC CA-3′. Dissociation kinetics was performed to check the specificity of oligonucleotides annealing. Standard curves were generated using a recombinant plasmid containing the full-length sequence of *AsOMT116*. The 18S ribosomal RNA expression was analyzed as an internal standard to normalize the transcript abundance in each sample. For the analysis of 18S ribosomal RNA expression, we used the TaqMan Ribosomal RNA Control Reagents VIC Probe kit (Applied Biosystems) according to the manufacturer's protocol at default parameters.

## Results

### *OMT activity in plant protein preparations from* A. sylvestris *organs*

Endogenous enzymatic activity responsible for TJOMT was investigated using crude proteins prepared from *A. sylvestris*. Crude protein assays with thujaplicatin and 5-*O*-methylthujaplicatin produced 5-*O*-methylthujaplicatin and 4,5-*O*,*O*-dimethylthujaplicatin, respectively, as confirmed by GC-MS analysis (Figures 2 and 3). The specific activities of crude proteins from different plant organs for thujaplicatin and 5-*O*-methylthujaplicatin, in which thujaplicatin was converted into 5-*O*-methylthujaplicatin and 5-*O*-methylthujaplicatin was converted to 4,5-*O*,*O*-dimethylthujaplicatin, were determined (Figure 4).

Specific activity for thujaplicatin was highest in assays incubated with the proteins obtained from *A. sylvestris* roots (Figure 4). On the other hand, specific activity for 5-*O*-methylthujaplicatin was highest in assays of the proteins obtained from stems. There were no significant differences in specific activities between complete and control assays for the following: thujaplicatin assays with proteins from young shoot; 5-*O*-methylthujaplicatin assays with proteins from root; 5-*O*-methylthujaplicatin assays with proteins from leaf; and 5-*O*-methylthujaplicatin assays with proteins from young shoot. The addition of $MgCl_2$ to the reaction did not affect the amount of product that was produced in plant protein assays with thujaplicatin or 5-*O*-methylthujaplicatin as the substrate (Table 2).

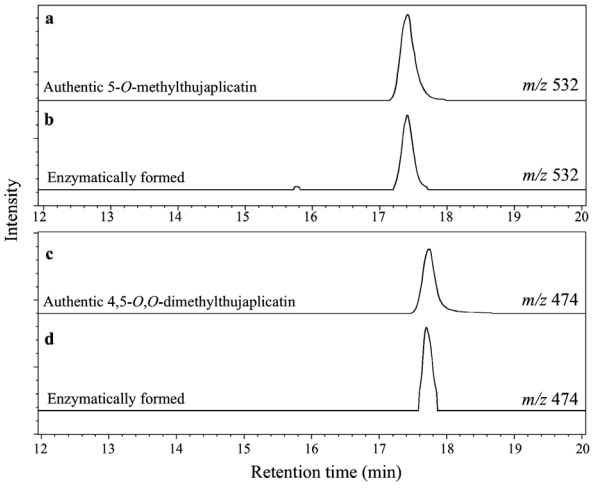


Figure 2. Selected-ion monitoring chromatograms of 5-*O*-methylthujaplicatin (a) and 4,5-*O*,*O*-dimethylthujaplicatin (c) authentic samples, and of plant protein assay products when incubated with thujaplicatin (b) and 5-*O*-methylthujaplicatin (d).

Table 2. *A. sylvestris* plant protein assays.

| Assay type | $V$ (nmol min$^{-1}$ mg$^{-1}$) | |
|---|---|---|
| | Root-thujaplicatin* | Stem-5-*O*-methylthujaplicatin* |
| Complete assay with $Mg^{2+}$ | $0.550 \pm 6.5 \times 10^{-3}$ | $9.68 \times 10^{-3} \pm 1.04 \times 10^{-3}$ |
| Complete assay | $0.533 \pm 2.0 \times 10^{-2}$ | $9.10 \times 10^{-3} \pm 3.73 \times 10^{-4}$ |
| Control (boiled protein) | $0.003 \pm 1.2 \times 10^{-5}$ | $1.02 \times 10^{-3} \pm 6.82 \times 10^{-5}$ |
| Control (no SAM) | $0.022 \pm 1.9 \times 10^{-4}$ | $3.04 \times 10^{-3} \pm 1.47 \times 10^{-4}$ |
| Control (no lignan substrate) | $0.002 \pm 1.8 \times 10^{-4}$ | $1.47 \times 10^{-3} \pm 9.59 \times 10^{-5}$ |

* Plant proteins were extracted from roots and stem for thujaplicatin and 5-*O*-methylthujaplicatin assays, respectively.

Table 3. Deduced protein properties of the isolated *AsOMT* cDNAs.

| cDNA | DDBJ accession number | ORF (nucleotides) | Deduced molecular mass (kDa) | Deduced isoelectric point (pI) |
|---|---|---|---|---|
| *AsOMT15* | AB820125 | 1074 | 39.46 | 6.09 |
| *AsOMT29* | AB820126 | 1098 | 40.18 | 5.83 |
| *AsOMT50* | AB820127 | 1062 | 39.27 | 5.51 |
| *AsOMT58* | AB820128 | 1098 | 40.14 | 5.62 |
| *AsOMT95* | AB820129 | 1062 | 38.63 | 5.39 |
| *AsOMT116* (*AsTJOMT*) | AB820130 | 1110 | 40.11 | 4.94 |
| *AsOMT148* | AB820131 | 1080 | 39.01 | 5.33 |

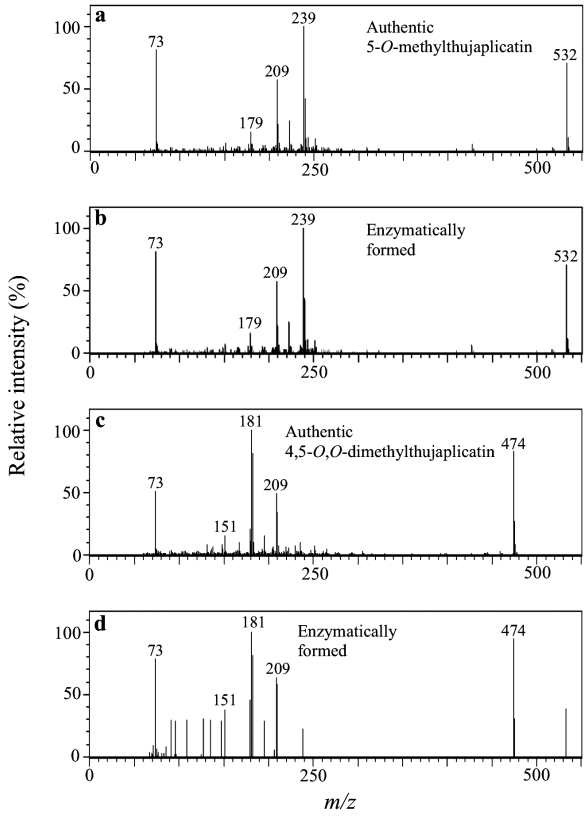


Figure 3. Mass spectra of authentic 5-*O*-methylthujaplicatin (a), plant protein assay product after incubation with thujaplicatin (b), authentic 4,5-*O*,*O*-dimethylthujaplicatin (c), and plant protein assay product after incubation with 5-*O*-methylthujaplicatin (d). Plant protein was extracted from *A. sylvestris* roots (thujaplicatin assays) or stems (5-*O*-methylthujaplicatin assays).

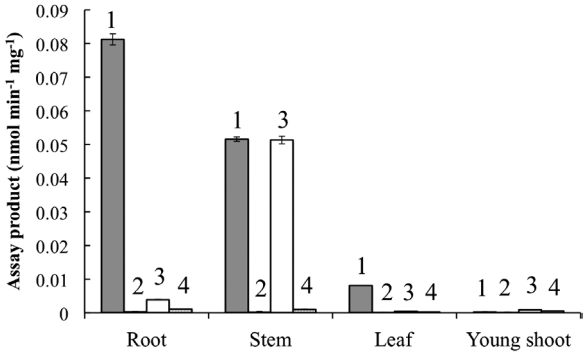


Figure 4. Specific activities of protein extracted from *A. sylvestris* organs with the following substrates: (1) thujaplicatin, (2) thujaplicatin (boiled plant protein), (3) 5-*O*-methylthujaplicatin, and (4) 5-*O*-methylthujaplicatin (boiled plant protein). Specific activity for AsTJOMT in roots was significantly higher than in other organs (one-way ANOVA, $p<0.001$, $n=3$).

### *Isolation of cDNAs encoding* O*-methyltransferases*

A cDNA library was constructed from mRNAs prepared from *A. sylvestris* roots and young shoots. The cDNA library was screened with a probe mixture containing four plant *OMT* cDNAs. To obtain a range of *AsOMT* cDNAs, these probes were selected from different *OMT* subfamilies. One hundred and sixty three positive clones were obtained by screening, which were divided into 21 molecular species of truncated *OMT*s. Walking experiments confirmed the ORFs of the *OMT*s and showed seven distinct *AsOMT* cDNAs (Table 3). The cDNAs were submitted to the DNA Data Bank of Japan (DDBJ), and their accession numbers are shown in Table 3.

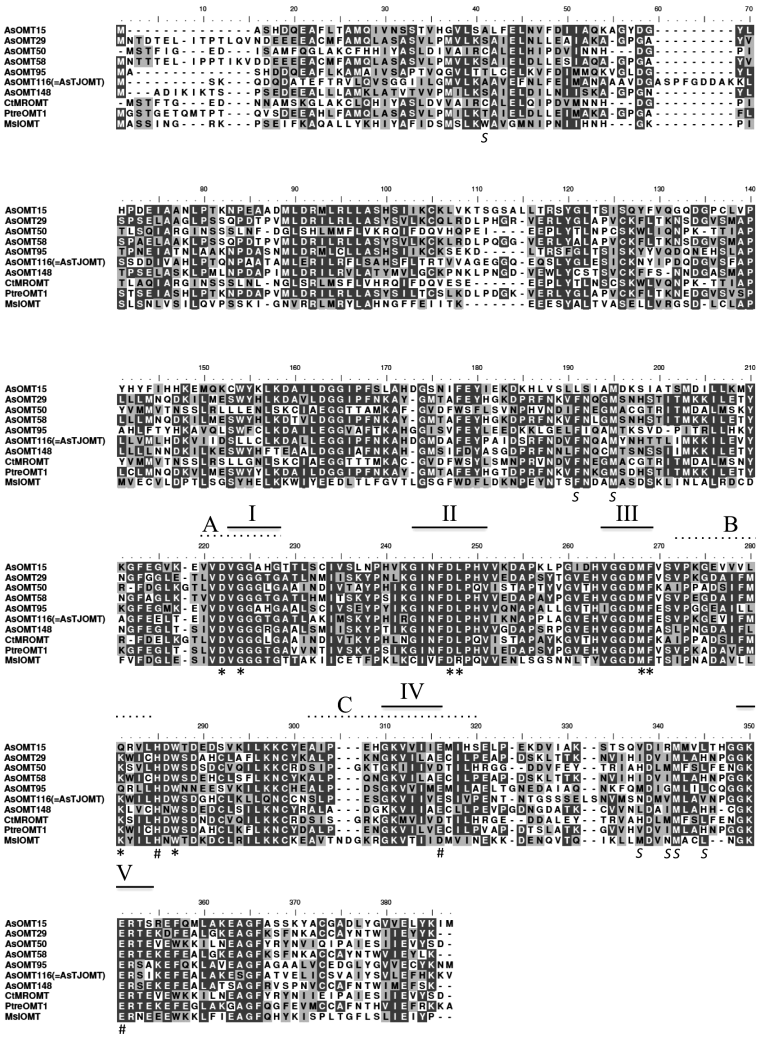


Figure 5. Alignment of the predicted amino acid sequence of the AsOMTs and related OMTs. SAM-binding motifs A, B, and C and regions I, II, III, IV, and V are domains conserved among plant OMTs (Dunlevy et al. 2010; Joshi and Chiang 1998; Umezawa et al. 2013). Catalytic (#), SAM binding (*), and substrate-binding (*S*) residues were determined from the *Medicago sativa* isoflavone OMT (Zubieta et al. 2001).

### In silico *phylogenetic analysis of the AsOMTs*

*OMT* cDNAs isolated in this research had high similarity to previously characterized plant *OMT*s, in terms of their deduced polypeptide lengths, conserved motifs (Ibrahim et al. 1998; Joshi and Chiang 1998) (Figure 5), deduced molecular masses, and deduced isoelectric points (Table 3). After alignment by MAFFT at the amino acid level, a phylogenetic tree generated by the neighbor-joining method showed that AsOMT15 and AsOMT95 were most closely related to the *Pimpinella anisum t*-anol/isoeugenol OMT (Koeduka et al. 2009); AsOMT29 and AsOMT58 were most closely related to typical plant CAldOMTs (Bugos et al. 1991; Ibrahim et al. 1998; Joshi and Chiang 1998); AsOMT50, AsOMT116 and AsOMT148 were most closely related to CtMROMT (Umezawa et al. 2013), the *Rosa chinensis* phloroglucinol OMT (Wu et al. 2004) and the *Ammi majus* bergaptol OMT (Hehmann et al. 2004), respectively (Figure 6).

### *Expression and preliminary enzymatic analysis of recombinant AsOMT116*

Recombinant OMTs were produced in *E. coli* BL21 (DE3) cells. Preliminary enzymatic assays of crude lysate and selected substrates showed that out of the seven putative OMTs, only three had OMT activity: recombinant AsOMT29 accepted caffeic acid and 5-hydroxyferulic acid as substrates; AsOMT50 and AsOMT116 accepted matairesinol and thujaplicatin as a substrate, respectively. In this paper, we characterized recombinant AsOMT116 that methylated thujaplicatin, which is a biosynthetic precursor of yatein (Sakakibara et al. 2003, Figure 1). The characterization of the other OMTs are currently underway.

### *Regioselectivity of recombinant AsOMT116-catalyzed reactions*

SDS-PAGE analysis of the purified recombinant AsOMT116 showed apparent homogeneity (Figure 7)

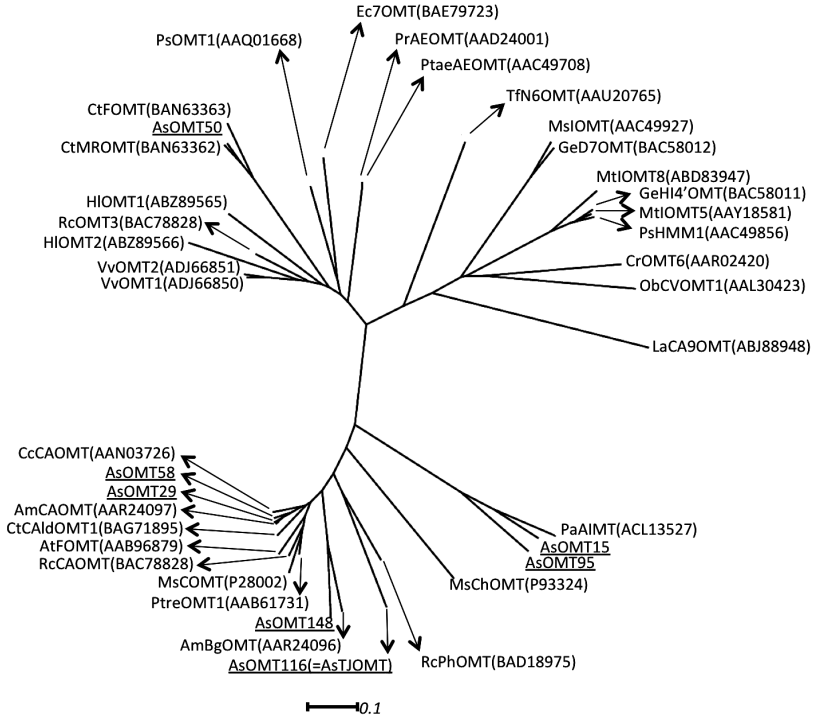


Figure 6. Phylogenetic tree showing relationship of AsOMTs with previously reported plant OMTs. GenBank accession numbers are in parentheses after protein names.

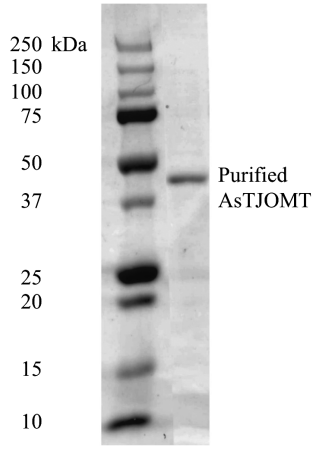


Figure 7. SDS-PAGE of AsTJOMT purification by His-bind affinity resin chromatography. The single band shows that AsTJOMT was successfully purified to homogeneity.

at around 40 kDa, which is consistent with the deduced molecular mass of AsOMT116. Of all the substrates that we tested, AsOMT116 specifically methylated thujaplicatin. GC-MS analysis showed that recombinant AsOMT116 enzyme assays with thujaplicatin gave a product peak at retention time, 20.23 min. By comparing the AsOMT116 assay product's retention time and mass spectrum (Figure 8a) with those of the authentic samples [5-*O*-methylthujaplicatin: retention time, 20.23 min, mass spectrum, Figure 8b; 4-*O*-methylthujaplicatin: retention time, 20.13 min, mass spectrum, Figure 8d; 4′-*O*-methylthujaplicatin: retention time, 18.22 min,

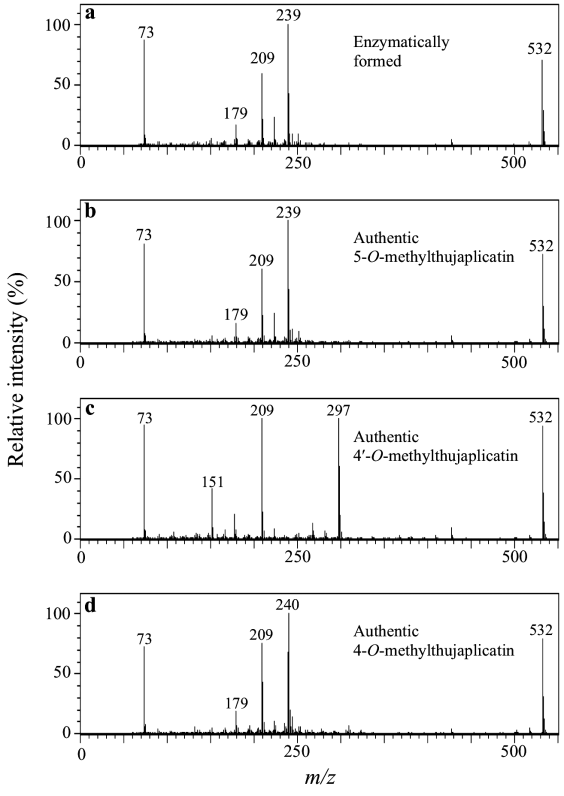


Figure 8. Mass spectrum of AsTJOMT assay product after incubation with thujaplicatin (a) compared with those of authentic 5-*O*-methylthujaplicatin (b), 4′-*O*-methylthujaplicatin (c), and 4-*O*-methylthujaplicatin (d). From the mass spectrum and retention time comparisons, the product of the AsTJOMT-mediated methylation product of thujaplicatin is unequivocally 5-*O*-methylthujaplicatin.

mass spectrum, Figure 8c], we were able to confirm that AsOMT116 catalyzes methylation of the hydroxyl group at the 5-position of thujaplicatin's aromatic ring to produce 5-*O*-methylthujaplicatin. Thus, *AsOMT116* was re-designated *A. sylvestris thujaplicatin OMT* (*AsTJOMT*).

### *Kinetic analysis of AsTJOMT*

For further characterization, we analyzed the kinetic parameters of AsTJOMT for its substrate, thujaplicatin. The purified recombinant AsTJOMT was analyzed by GC-MS to determine its $K_m$, $V_{max}$, and $k_{cat}$ values. The assays were done in potassium phosphate buffer (pH 7) at 30°C for 15 min. Lineweaver–Burk analysis revealed that the $K_m$ and $V_{max}$ values for AsTJOMT were 3.8 $\mu$M and 8.71 pmol min$^{-1}$, respectively, when 1.21 $\mu$g of the recombinant enzyme was used in the assay. Assuming that AsTJOMT was a monomer, the $E_t$ value was calculated by dividing the amount of recombinant AsTJOMT in the reaction (1.21 $\mu$g) by its deduced molecular mass (40,110 Da), resulting in 0.03 nmol. Thus, the $k_{cat}$ value ($V_{max}/E_t$) for AsTJOMT was 0.29 min$^{-1}$. The catalytic efficiency ($k_{cat}/K_m$) of AsTJOMT was 0.076 min$^{-1}$ $\mu$M$^{-1}$.

### *Gene expression analysis of* AsTJOMT *in different organs*

The expression levels of *AsTJOMT* in several organs were elucidated by qRT-PCR. The organs tested included young shoots, leafs, roots, and stems. The expression level of the gene was highest in the root samples (one-way ANOVA, $p<0.001$, $n=3$). The lowest *AsTJOMT* expression level was observed in young shoot samples (Figure 9).

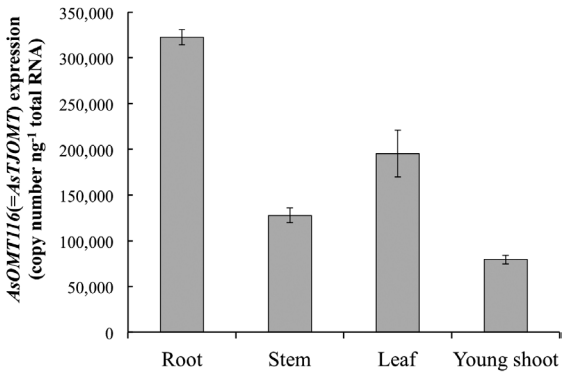


Figure 9. Quantification of *AsTJOMT* expression levels in various *A. sylvestris* organs. Expression of the gene was highest in roots (one-way ANOVA, $p<0.001$, $n=3$). This result is congruent with metabolite analysis by Koulman et al. (2003), which showed the highest yatein accumulation in roots.

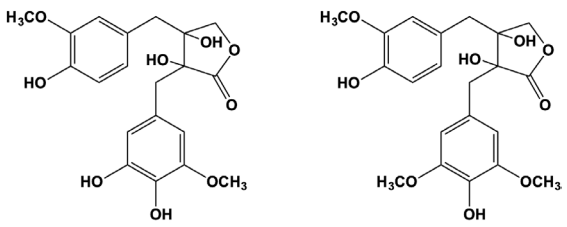


Figure 10. Lignans in the genus *Thuja* (Kutsuki et al. 1981).

## Discussion

Kutsuki et al. (1981) reported that *Thuja* OMTs could not catalyze the methylation of dihydroxythujaplicatin to its methyl-ether derivative (Figure 10). Through feeding experiments, Sakakibara et al. (2003) demonstrated that thujaplicatin is converted into its methyl-ether derivative through OMT catalysis giving rise to 5-*O*-methylthujaplicatin (Figure 1). In the present study, we confirmed OMT activity in crude *A. sylvestris* proteins towards thujaplicatin and 5-*O*-methylthujaplicatin, giving rise to their methyl-ether products.

We isolated seven cDNAs encoding putative AsOMTs through DIG-based screening. As a comparison to the traditional screening method, we also conducted next generation sequencing, where we isolated all except two of the cDNAs that were identified using the DIG-screening method (*AsOMT50* and *AsOMT148*) (data not shown). The recombinant protein of *AsOMT116* was able to catalyze regioselective methylation of thujaplicatin at the 5-position of the aromatic ring, producing 5-*O*-methylthujaplicatin, and was therefore designated *A. sylvestris* thujaplicatin (AsTJ) OMT. This result also indicates that AsTJOMT is responsible for the first methylation step in *A. sylvestris* yatein biosynthesis from matairesinol as proposed by Sakakibara et al. (2003) (Figure 1). The gene responsible for the second methylation step of the proposed yatein biosynthesis pathway in *A. sylvestris*, involving methylation at the 4-position of the aromatic ring of 5-*O*-methylthujaplicatin to produce 4,5-*O*,*O*-dimethylthujaplicatin is yet to be elucidated.

The result of the gene expression analysis of several *A. sylvestris* tissues showed that the expression level of *AsTJOMT* was significantly higher in roots than in other organs. Furthermore, plant protein assays showed that specific activity for thujaplicatin was highest in assays of protein extracted from roots. This is in line with a previous study, in which lignans such as yatein and deoxypodophyllotoxin, whose biosynthesis involves AsTJOMT, were found at high amounts in *A. sylvestris* roots (Koulman et al. 2003), strongly indicating that *A. sylvestris* roots are important for both lignan biosynthesis and storage.

The characterization of genes that are related in the lignan biosynthesis pathway is important for biotechnological production of chemicals in the

pharmaceutical industry. In this research, we have isolated and characterized an *OMT* cDNA that is responsible for the biosynthesis of the lignan yatein, a precursor to podophyllotoxin.

To the best of our knowledge, this is the first report of a cDNA encoding a thujaplicatin specific OMT that catalyzes regiospecific methylation at the 5-position of the thujaplicatin aromatic ring. Elucidation of the OMT related in the *O*-methylation of 5-*O*-methylthujaplicatin into 4,5-*O*,*O*-dimethylthujaplicatin, the second *O*-methylation step in the *A. sylvestris* yatein biosynthetic pathway from matairesinol, is also an interesting subject as a follow up to this research.

## Acknowledgements

This research was partly supported by a grant from the New Energy and Industrial Technology Development Organization (Development of Fundamental Technologies for Controlling the Process of Material Production of Plants), by Grants-in-Aid from the Japan Society for the Promotion of Science (nos. 12660150, 16380116, and 18658069), by a grant from Suntory Co. Ltd., and by a grant from the Institute of Sustainability Science, Kyoto University. Thanks are also due to Dr. Daisuke Shibata at Kazusa DNA Research Institute for performing the next generation sequencing. A part of this study was conducted using the Forest Biomass Analytical System (FBAS), at the Research Institute for Sustainable Humanosphere, Kyoto University. The authors thank Ashiu Forest Research Station, Field Science Education and Research Center, Kyoto University, Japan, for providing *Anthriscus sylvestris* plants.

## References

Bugos RC, Chiang VL, Campbell WH (1991) cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of aspen. *Plant Mol Biol* 17: 1203–1215

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep* 11: 113–116

Dunlevy JD, Soole KL, Perkins MV, Dennis EG, Keyzers RA, Kalua CM, Boss PK (2010) Two *O*-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavour. *Plant Mol Biol* 74: 77–89

Farkya S, Bisaria VS, Srivastava AK (2004) Biotechnological aspects of the production of the anticancer drug podophyllotoxin. *Appl Microbiol Biotechnol* 65: 504–519

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) *The Proteomics Protocols Handbook*. Humana Press, Totowa, New Jersey, pp 571-607

Hall TA (1999) Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95–98

Hehmann M, Lukačin R, Ekiert H, Matern U (2004) Furanocoumarin biosynthesis in *Ammi majus* L. Cloning of bergaptol *O*-methyltransferase. *Eur J Biochem* 271: 932–940

Huang X (1992) A contig assembly program based on sensitive detection of fragment overlaps. *Genomics* 14: 18–25

Ibrahim RK, Bruneau A, Bantignies B (1998) Plant *O*-methyltransferases: molecular analysis, common signature and classification. *Plant Mol Biol* 36: 1–10

Ikeda R, Nagao T, Okabe H, Nakano Y, Matsunaga H, Katano M, Mori M (1998) Antiproliferative constituents in Umbelliferae plants. III. constituents in the root and the ground part of *Anthriscus sylvestris* HOFFM. *Chem Pharm Bull* 46: 871–874

Jeong G-S, Kwon O-K, Park B-Y, Oh S-R, Ahn K-S, Chang M-J, Oh WK, Kim J-C, Min B-S, Kim Y-C, et al. (2007) Lignans and coumarins from the roots of *Anthriscus sylvestris* and their increase of caspase-3 activity in HL-60 cells. *Biol Pharm Bull* 30: 1340–1343

Joshi CP, Chiang VL (1998) Conserved sequence motifs in plant *S*-adenosyl-L-methionine-dependent methyltransferases. *Plant Mol Biol* 37: 663–674

Kadkade PG (1982) Growth and podophyllotoxin production in callus tissues of *Podophyllum peltatum*. *Plant Sci Lett* 24: 107–115

Katoh K, Asimenos G, Toh H (2009) Multiple alignment of DNA sequences with MAFFT. In: Posada D (ed) *Bioinformatics for DNA sequence analysis. Methods in molecular biology*. Vol 537. Humana Press, Totowa, New Jersey, pp 39–64

Koeduka T, Baiga TJ, Noel JP, Pichersky E (2009) Biosynthesis of *t*-anethole in anise: characterization of *t*-anol/isoeugenol synthase and an *O*-methyltransferase specific for a C7–C8 propenyl side chain. *Plant Physiol* 149: 384–394

Koulman A, Kubbinga ME, Batterman S, Woerdenbag HJ, Pras N, Woolley JG, Quax WJ (2003) A phytochemical study of lignans in whole plants and cell suspension cultures of *Anthriscus sylvestris*. *Planta Med* 69: 733–738

Kozawa M, Baba K, Matsuyama Y, Kido T, Sakai M, Takemoto T (1982) Components of the root of *Anthriscus sylvestris* HOFFM. II. insecticidal activity. *Chem Pharm Bull* 30: 2885–2888

Kutsuki H, Shimada M, Higuchi T (1981) The role of *O*-methyltransferase in the formation of lignin and lignans in the genus *Thuja*. *Mokuzai Gakkaishi* 27: 39–48

Li L, Popko JL, Zhang X-H, Osakabe K, Tsai C-J, Joshi CP, Chiang VL (1997) A novel multifunctional *O*-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. *Proc Natl Acad Sci USA* 94: 5461–5466

Li L, Popko JL, Umezawa T, Chiang VL (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J Biol Chem* 275: 6537–6545

Lim Y-H, Leem M-J, Shin D-H, Chang H-B, Hong S-W, Moon E-Y, Lee D-K, Yoon S-J, Woo W-S (1999) Cytotoxic constituents from the roots of *Anthriscus sylvestris*. *Arch Pharm Res* 22: 208–212

Marques JV, Kim K-W, Lee C, Costa MA, May GD, Crow JA, Davin LB, Lewis NG (2013) Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. *J Biol Chem* 288: 466–479

Nakatsubo T, Li L, Hattori T, Lu S, Sakakibara N, Chiang VL, Shimada M, Suzuki S, Umezawa T (2007) Roles of 5-hydroxyconiferylaldehyde and caffeoyl CoA *O*-methyltransferases in monolignol biosynthesis in *Carthamus tinctorius*. *Cellul Chem Technol* 41: 511–520

Nakatsubo T, Kitamura Y, Sakakibara N, Mizutani M, Hattori T, Sakurai N, Shibata D, Suzuki S, Umezawa T (2008a) *At5g54160* gene encodes *Arabidopsis thaliana* 5-hydroxyconiferaldehyde *O*-methyltransferase. *J Wood Sci* 54: 312–317

Nakatsubo T, Mizutani M, Suzuki S, Hattori T, Umezawa T (2008b)

Characterization of *Arabidopsis thaliana* pinoresinol reductase, a new type of enzyme involved in lignan biosynthesis. *J Biol Chem* 283: 15550–15557

Ono E, Nakai M, Fukui Y, Tomimori N, Fukuchi-Mizutani M, Saito M, Satake H, Tanaka T, Katsuta M, Umezawa T, et al. (2006) Formation of two methylenedioxy bridges by a *Sesamum* CYP81Q protein yielding a furofuran lignan, (+)-sesamin. *Proc Natl Acad Sci USA* 103: 10116–10121

Sakakibara N, Suzuki S, Umezawa T, Shimada M (2003) Biosynthesis of yatein in *Anthriscus sylvestris*. *Org Biomol Chem* 1: 2474–2485

Sakakibara N, Nakatsubo T, Suzuki S, Shibata D, Shimada M, Umezawa T (2007) Metabolic analysis of the cinnamate/monolignol pathway in *Carthamus tinctorius* seeds by a stable-isotope-dilution method. *Org Biomol Chem* 5: 802–815

Schröder G, Wehinger E, Schröder J (2002) Predicting the substrates of cloned plant *O*-methyltransferases. *Phytochemistry* 59: 1–8

Seidel V, Windhövel J, Eaton G, Alfermann AW, Arroo RRJ, Medarde M, Petersen M, Woolley JG (2002) Biosynthesis of podophyllotoxin in *Linum album* cell cultures. *Planta* 215: 1031–1039

Suzuki S, Umezawa T (2007) Biosynthesis of lignans and norlignans. *J Wood Sci* 53: 273–284

Umezawa T (2003) Diversity in lignan biosynthesis. *Phytochem Rev* 2: 371–390

Umezawa T (2010) The cinnamate/monolignol pathway. *Phytochem Rev* 9: 1–17

Umezawa T, Ragamustari SK, Nakatsubo T, Wada S, Li L, Yamamura M, Sakakibara N, Hattori T, Suzuki S, Chiang VL (2013) A novel lignan *O*-methyltransferase catalyzing the regioselective methylation of matairesinol in *Carthamus tinctorius*. *Plant Biotechnol* 30: 97–109

Van Uden W, Bos JA, Boeke GM, Woerdenbag HJ, Pras N (1997) The large-scale isolation of deoxypodophyllotoxin from rhizomes of *Anthriscus sylvestris* followed by its bioconversion into 5-methoxypodophyllotoxin $\beta$-D-glucoside by cell cultures of *Linum flavum*. *J Nat Prod* 60: 401–403

Wu S, Watanabe N, Mita S, Dohra H, Ueda Y, Shibuya M, Ebizuka Y (2004) The key role of phloroglucinol *O*-methyltransferase in the biosynthesis of *Rosa chinensis* volatile 1,3,5-trimethoxybenzene. *Plant Physiol* 135: 95–102

Zubieta C, He X-Z, Dixon RA, Noel JP (2001) Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. *Nat Struct Biol* 8: 271–279