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Research

The O-methyltransferase PMT2 mediates methylation of pinosylvin in Scots pine

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Summary

- Heartwood extractives are important determinants of the natural durability of pine heartwood. The most important phenolic compounds affecting durability are the stilbenes pinosylvin and its monomethylether, which in addition have important functions as phytoalexins in active defense. A substantial portion of the synthesized pinosylvin is 3-methoxylated but the *O*-methyltransferase responsible for this modification has not been correctly identified.
- We studied the expression of the stilbene pathway during heartwood development as well as in response to wounding of xylem and UV-C treatment of needles. We isolated and enzymatically characterized a novel *O*-methyltransferase, PMT2. The methylated product was verified as pinosylvin monomethylether using ultra performance liquid chromatography–tandem mass spectrometry and high performance liquid chromatography analyses.
- The PMT2 enzyme was highly specific for stilbenes as substrate, in contrast to caffeoyl-CoA *O*-methyltransferase (CCoAOMT) and PMT1 that were multifunctional. Expression profile and multifunctional activity of CCoAOMT suggest that it might have additional roles outside lignin biosynthesis. PMT1 is not involved in the stilbene pathway and its biological function remains an open question.
- We isolated a new specific *O*-methyltransferase responsible for 3-methoxylation of pinosylvin. Expression of PMT2 closely follows stilbene biosynthesis during developmental and stress induction. We propose that PMT2 is responsible for pinosylvin methylation in Scots pine (*Pinus sylvestris*), instead of the previously characterized methyltransferase, PMT1.

Introduction

The phenylpropanoid pathway provides metabolites for several essential functions from structural support to defense and pigmentation. Lignin, flavonoids and stilbenes are all formed from different branches of the same pathway (Fig. 1). Lignin and flavonoids are found in all vascular plants, whereas stilbenes are found sporadically in genera like *Vitis*, *Pinus* and *Arachis* (Chong *et al.*, 2009). Lignin is a polyphenolic polymer that has a vital role in strengthening plant cell walls but at the same time it interferes with utilization of woody biomass. In conifers lignin is formed from *p*-coumaryl alcohol (H units) and coniferyl alcohol (G units), and lacks sinapyl alcohol (S units), which are common in angiosperm wood (Fig. 1). The pine stilbenes pinosylvin (3,5-dihydroxystilbene, PS) and its monomethylether (3-methoxy-5-hydroxystilbene, PSME) are phenolic secondary metabolites that play important roles in inducible and constitutive defense in pines. During heartwood formation, stilbenes accumulate in the heartwood where they protect the wood against decaying fungi (Harju & Venäläinen, 2006). Different abiotic and biotic stresses like UV-C (Schoeppner & Kindl, 1979), wounding (Harju *et al.*, 2009), ozone (Rosemann *et al.*, 1991), herbivores (Sullivan *et al.*, 1992) and pathogens (Gehlert *et al.*, 1990) activate the production of stilbenes also in the sapwood and needles where they therefore function as phytoalexins. Stilbenes are widely studied not only for their importance to plants, but also because of their beneficial effects on human health. PS has been shown to have positive effects in preventing cancer and cardiovascular diseases (Park *et al.*, 2012; Jeong *et al.*, 2013), has anti-inflammatory properties (Laavola *et al.*, 2015) and because of their wide antimicrobial activity, both PS and PSME have potential uses as food preservatives (Plumed-Ferrer *et al.*, 2013).

The diversity of plant-produced secondary metabolites is greatly increased by enzymatic modifications of side groups of common structural backbones, thereby altering the properties of the compounds. Methylation of hydroxyl side groups is an important modification in plants and other organisms, and is catalyzed by *O*-methyltransferases (OMTs) that use *S*-adenosyl-L-methionine (SAM) as a methyl group donor. Methylation of reactive hydroxyl groups alters the solubility and reactivity of compounds and may consequently affect, for example, their antimicrobial activity (Lam *et al.*, 2007). For instance, pterostilbene, a dimethylated derivative of resveratrol inhibits fungal growth five times more strongly than the unmethylated resveratrol (Jeandet *et al.*, 2002). However, PSME seems to be less toxic to fungi in general than PS although it has stronger

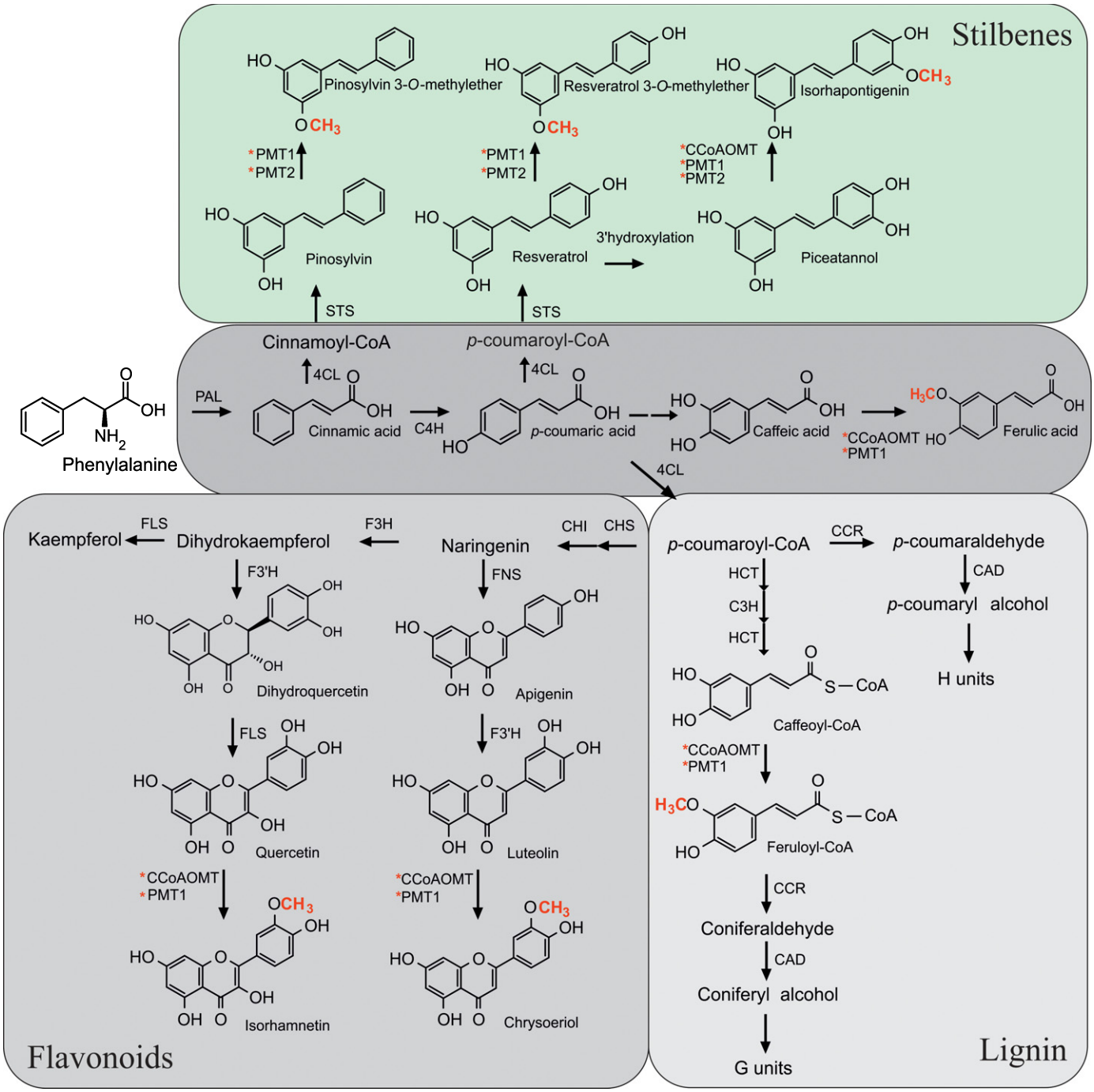

Fig. 1 Biosynthetic pathways to stilbenes, flavonoids and lignin, shown for parts relevant for this study. Enzymatic methylation reactions tested are marked with red asterisks and the attached methyl groups are marked in red. PMT, pinosylvin *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; STS, stilbene synthase; 4CL, *p*-coumaroyl-CoA ligase; PAL, phenylalanine ammonia-lyase; FLS, flavonol synthase; F3H, flavanone 3-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; CCR, cinnamoyl-CoA reductase; F3′H, flavonoid 3′-hydroxylase; FNS, flavone synthase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CAD, cinnamyl alcohol dehydrogenase; C3H, coumarate 3-hydroxylase.

activity against some specific brown-rot fungi (Hart, 1981). In heartwood of Scots pine (*Pinus sylvestris*), more than half of PS is methylated into PSME (Venäläinen *et al.*, 2004).

Plant OMTs are divided into two subgroups (Noel *et al.*, 2003; Louie *et al.*, 2010). Type I OMTs form a functionally divergent group and methylate a wide variety of substrates such as flavonoids, alkaloids and stilbenes. They are *c.* 360 amino acids in length, function as homodimers and their catalytic activity is not dependent on metal ions. From the characterized plant type I OMTs some are substrate specific (Nagel *et al.*, 2008) and some have been shown to be multifunctional accepting several substrates (Chiron *et al.*, 2000a; Zubieta *et al.*, 2002; Lam *et al.*, 2007). A type I multifunctional *O*-methyltransferase PMT, with pinosylvin methylating activity, here referred to as PMT1, has

been described from Scots pine (*P. sylvestris*) by Chiron *et al.* (2000a). In addition to PS, the enzyme methylated several different stilbene aglycones, flavonoids, hydroxycinnamic acids and caffeoyl-CoA. Another multifunctional type I *O*-methyltransferase, AEOMT (hydroxycinnamic acids/hydroxycinnamoyl CoA esters OMT) was characterized from loblolly pine (*Pinus taeda*). AEOMT had similar specific activities for methylation of the lignin biosynthesis related substrates caffeic acid, 5-hydroxyferulic acid, caffeoyl-CoA and 5-hydroxyferuloyl-CoA, but it was inactive with flavonoid substrates (Li *et al.*, 1997). However, later studies showed that the enzyme is not coexpressed with lignin pathway genes in lignin-producing conditions, and most likely does not have a role in lignin biosynthesis (Anterola *et al.*, 2002).

Type II OMTs are of smaller molecular weight and dependent on divalent cations (Noel *et al.*, 2003; Louie *et al.*, 2010). A type II enzyme, CCoAOMT (caffeoyl-CoA *O*-methyltransferase), involved in methylation of caffeoyl-CoA in the biosynthesis of G-type lignin has been described in *P. taeda* (Li *et al.*, 1999). Downregulation of the enzyme in radiata pine (*Pinus radiata*) tracheary element cultures reduced lignin content by up to 20%, suggesting involvement of the enzyme in lignin biosynthesis (Wagner *et al.*, 2011).

Here, we describe characterization of a novel PS-specific type I *O*-methyltransferase enzyme, PMT2, from Scots pine and provide evidence that it is the true enzyme in the stilbene pathway instead of the previously characterized PMT1. We isolated also cDNA molecules encoding other known *O*-methyltransferases, AEOMT and CCoAOMT, from pine. We found that the pine CCoAOMT is, like PMT1, a multifunctional enzyme and that it might also have a role in methylating compounds outside the lignin biosynthesis pathway according to its expression profile and *in vitro* enzymatic activity. The AEOMT, although produced in a soluble form, did not show any activity in our *in vitro* enzymatic assays.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com) unless stated otherwise. For high performance liquid chromatography (HPLC), Fisher (www.fishersci.com) HPLC grade chemicals were used. Substrates for enzymatic assays were from TransMIT (www.transmit.de), except pinosylvin (3,5-dihydroxystilbene, PS) and its monomethylether (3-methoxy-5-hydroxystilbene, PSME) from ArboNova (www.arbonova.com), and isorhamnetin, piceatannol and rhapontigenin from Santa Cruz Biotechnology (www.scbt.com).

Plant material and treatments

For wounding experiments, 5-yr-old Scots pine seedlings were used. Seedlings were grown in the glasshouse and wounded by drilling through the stem as described by Harju *et al.* (2009). Xylem samples were collected 3 h (3H), 24 h (1D) and 96 h (4D) after wounding. Bark was removed and samples were frozen in liquid nitrogen and stored at −80°C.

For UV treatments, pine seedlings were grown in peat : vermiculite (1 : 1) under 16 h : 8 h, light : dark in controlled growth chambers at 20–22°C. Six-week-old seedlings were treated for 15 min with UV-C light from an uncovered mercury lamp (sterilAir UVC G9). Needles from 10 plants were collected and pooled together 2 h (2H), 6 h (6H) and 24 h (24H) after the onset of UV light. Samples were frozen in liquid nitrogen and stored at −80°C.

Sapwood (SW) and transition zone (TZ) samples were collected from *c.* 46-yr-old trees (Lim *et al.*, 2016).

RNA sequencing and gene expression analysis using RT-qPCR

Pine transcriptomes were sequenced from wounded xylem (control, 3H, 1D and 4D) and UV-C-treated needles (control, 2H, 6H and 24H) of seedlings, and SW and TZ of adult trees using the SOLiD platform. From each sample point four biological replicates were sequenced. Sample processing, sequencing and mapping the reads to the *Pinus* EST collection (The Gene Index Databases, v.9.0), Trinity assembly of the reads and to *Pinus taeda* L. genome v.1.01 was done as described in Lim *et al.* (2016). Mapped count data were used for correlation analysis to search for *O*-methyltransferases coexpressed with stilbene synthase. In the end, the genes cloned in this study encoding for pinosylvin *O*-methyltransferase (PMT2, PMT1), hydroxycinnamic acids/hydroxycinnamoyl CoA esters OMT (AEOMT) and caffeoyl-CoA *O*-methyltransferase (CCoAOMT) from Scots pine were mapped as described earlier and the counts normalized against the library size and transcript length.

Expression profiles of CCoAOMT, PMT1, PMT2 and stilbene synthase (STS) observed in RNA sequencing (RNA-seq) libraries were confirmed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the same RNA pools for cDNA synthesis that were used for RNA-seq. Preparation of RNA and cDNA samples is described later. Gene fragments were amplified using primers listed in Supporting Information Table S1. Actin and histone genes were used as references. Primer efficiencies were tested using a mixture of cDNA from UV-C 6H and wounding 2 day samples as a template except for STS, actin and histone primers that were tested earlier (Lim *et al.*, 2016). Amplification efficiencies were calculated using *EasyqpcR* R package module (Le Pape, 2015). Only primer pairs that had amplification efficiencies > 1.8 with SE < 5% were accepted. Samples from each time point of wounding and UV-C treatments and from SW and TZ samples were pooled together. In total, each time point of wounding series and SW and TZ samples had cDNA pooled from four individuals and every time point of UV-C treatment had cDNA from 40 individuals.

Reverse transcription quantitative polymerase chain reactions were carried out and data analyzed as described in Lim *et al.* (2016).

Isolation of enzyme coding cDNA molecules

Extraction of RNA from xylem, needle, transition zone and sapwood samples was done as described in Lim *et al.* (2016). A

quantity of 350, 500 and 800 ng of total RNA from TZ and SW, xylem and needle samples, respectively, was used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen; www.thermofisher.com) and oligo dT_{20} primer according to manufacturer's instructions. One microliter of the cDNA was used in each PCR reaction. Primers (listed in Table S1) were designed using the *P. taeda* sequences AF036095.1 for *CCoAOMT* and U39301.1 for *AEOMT*, and the *Pinus sylvestris* L. sequences X212322.1 for *PMT1* and TC166778 for *PMT2* as references. The primer binding areas were ensured by mapping SOLiD reads to the sequences to avoid any mismatches in the primer areas. *CCoAOMT* and *AEOMT* were amplified from control sample cDNA of the wounding experiment with primers CCoAOMTF and CCoAOMTR, and AEOMTF and AEOMTR, respectively. *PMT1* was amplified from 24H UV-C treated needles cDNA with primers PMT1F and PMT1R. *PMT2* was amplified from wounding 4D, UV-C 24H and transition zone cDNA using primers PMT2UTRF and PMT2UTRR that bound outside the protein coding region. PCR amplification was done using Phusion High-Fidelty polymerase (ThermoFisher Scientific; www.thermofisher.com) in a 20-μl reaction volume according to the manufacturer's instructions. PCR program was as following: 98°C 30 s, 30 cycles of 98°C 10 s, 61°C/61°C/59°C/60°C (for CCoAOMT/AEOMT/PMT1/PMT2, respectively) 30 s, 72°C 60 s, followed by 72°C 10 min. PCR products were ligated to pJET1.2 vector (ThermoFisher Scientific) according to manufacturer's instructions, transformed to *Escherichia coli* DH5α competent cells and sequenced. After sequencing, *PMT2* was further cloned for protein expression using primers PMT2_Flong and PMT2_R to create the longer version of the protein starting from the first ATG of the transcript and primers PMT2_Fshort and PMT2_R to create the short version starting from the second ATG. PCR amplification was done using Phusion High-Fidelty polymerase in a 20-μl reaction volume according the manufacturer's instructions using 50 ng of plasmid DNA as a template. PCR program was as following: 98°C 30 s, 15 cycles of 98°C 10 s, 61°C 30 s, 72°C 45 s, followed by 72°C 10 min. PCR products were ligated to the pJET1.2 vector, transformed to DH5α and sequenced.

Cloning of PMT2-encoding gene from haploid megagametophyte DNA

DNA was extracted from megagametophyte tissue of four seeds from a single Scots pine individual. DNA was extracted using E.Z.N.A® Plant DNA Kit (Omega bio-tek, Norcross, GA, USA) according to manufacturer's instructions. DNA was eluted with 100 μl of elution buffer. PMT2 was amplified as before using primers PMT2UTRF and PMT2UTRR and 2 μl of haploid DNA as template. PCR products were ligated to pJET1.2 vector, transformed to DH5α and sequenced.

Protein expression and purification

Protein coding sequences were isolated from the cloning vector pJET1.2 using restriction enzymes. AEOMT gene was inserted between *Sac*I and *Not*I, CCoAOMT between *Eco*RI and *Hin*dIII, PMT1 between *Hin*dIII and *Xho*I, and PMT2 between *Bam*HI and *Xho*I sites of the expression vector pHis8 (Jez *et al.*, 2000) to create N-terminally histidine tagged proteins. Constructs were sequenced in *E. coli* strain DH5α and subsequently transformed to the strain Lemo21(DE3) for expression (New England Biolabs; www.neb.com). Bacterial cultures were grown in LB medium containing 50 μg ml^{-1} kanamycin and 30 μg ml^{-1} chloramphenicol at 37°C until OD600 reached 0.3–0.6. Protein expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28°C for 5 h. Ten microliters of bacterial cells were pelleted and soluble proteins extracted with 600 μl of 1× LEW (Lysis-Equilibrium-Wash) buffer from a PrepEase His-Tagged Protein Purification Mini Kit (Affymetrix USB, Cleveland, OH, USA) supplemented with 0.5 mg of lysozyme, 10 U DNaseI and 1× cOmplete EDTA free protease inhibitor (Roche, Mannheim, Germany) by incubating at room temperature for 10–15 min with gentle shaking. Proteins were purified using nickel columns of the same kit according to manufacturer's instructions, except that the washing step was done once with binding buffer and twice with 5 mM imidazole in the binding buffer. Proteins were eluted with elution buffer that contained 250 mM of imidazole. The elution buffer was changed to the assay buffer 100 mM Tris-HCl pH 8.5 for PMT2, 100 mM Hepes pH 7.7 for PMT1 and 50 mM Tris-HCl pH 7.5 for CCoAOMT and AEOMT, each containing 10% of glycerol and 2 mM of dithiothreitol (DTT), with PD MiniTrap™ G-25 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) columns (Fig. S1). Protein concentrations were determined using Bradford assay (Biorad, Hercules, CA, USA) using BSA as standard.

Enzymatic assays

The pH optimum for PMT2 and CCoAOMT was estimated in Tris-HCl buffer for the pH range of 7.0–9.5, and for PMT1 in Tris-HCl for pH 7.0–9.5 and in HEPES for pH 7.0–8.5.

Enzymatic reaction mixtures for screening of potential substrates consisted of 5 μg of purified protein, 100 μM of substrate, 10 μM of ^{14}C labelled SAM (PerkinElmer, Boston, MA, USA) and a final concentration of 100 mM Tris-HCl pH 8.5 for PMT2, 100 mM Hepes pH 7.7 for PMT1 and 50 mM Tris-HCl pH 7.5 for CCoAOMT and AEOMT in a 100-μl reaction volume. Empty vector control samples included 5 μg of total proteins from the bacterial lysate in PMT2 assay conditions. In CCoAOMT reactions, a final concentration of 200 μM $MgCl_2$ was used. Reactions were pre-incubated for 5 min at 30°C without ^{14}C-SAM, started by adding SAM and incubated at 30°C for 30 min. All reactions except samples with caffeoyl-CoA were stopped with 20 μl of glacial acetic acid. Caffeoyl-CoA samples were stopped and hydrolysed by adding 11 μl of 5 M NaOH and incubated for 15 min at 40°C. After hydrolysis, 12.4 μl of 6 M HCl was added.

All samples were extracted twice with 250 μl of ethyl acetate, 3 ml of Optiphase HiSafe 3 (PerkinElmer) liquid scintillation cocktail was added to the combined extracts and radioactivity of the samples was measured for 5 min with a scintillation counter (Wallac 1414 WinSpectral v.1.40; Wallac Oy, Turku, Finland).

All the reactions were done in duplicate or triplicate and all the experiments were repeated at least once.

Kinetic parameters were determined in 50 μl reaction volumes that had 2.5 μg of purified protein, 195 μM of unlabeled SAM, 5 μM of ^{14}C-SAM, final concentrations of PS ranging from 5 to 200 μM and a final concentration of 100 mM Tris-HCl pH 8.5 for PMT2 and 100 mM Hepes pH 7.7 for PMT1. Reactions were pre-incubated for 5 min at 30°C without SAM, started by adding SAM and incubated at 30°C for 20 min. Samples were extracted and radioactivity measured as before. Kinetic constants were determined using nonlinear regression analysis, fit to the Michaelis-Menten equation (Prism7; GraphPad, La Jolla, CA, USA).

Enzymatic assays for HPLC and ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis were done in 200-μl reaction volumes that had 10–20 μg of purified protein, 200 μM of SAM, 100 μM of substrates in final concentration of 100 mM Tris-HCl pH 8.5 for PMT2 and 100 mM Hepes pH 7.7 for PMT1. The reaction was incubated at 30°C for 1 h. All reactions except caffeoyl-CoA were stopped with glacial acetic acid. Caffeoyl-CoA reactions were hydrolysed as described earlier. Samples were extracted twice with 500 μl of ethylacetate, evaporated to dryness and resultant residue was dissolved in 50 μl of methanol. Two reactions were combined for HPLC analysis and three samples for UPLC-MS/MS analysis.

HPLC and UPLC-MS/MS analyses

HPLC analyses were carried out on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) with Kinetex EVO C-18: 250 × 4.6 mm, 5 μM column (Phenomenex, Torrance, CA, USA). Chromatographic separation was done with linear gradient run of 20 min from 10% acetonitrile with 0.08% TFA to 90% acetonitrile with 0.08% trifluoroacetic acid (TFA), with flow rate of 1 ml min^{-1} at 35°C and 15 min of equilibration between the samples with a linear gradient to reach the starting conditions. Injection volume of samples was 20 μl. Detection was done with diode array detector (DAD) at the wavelength of 276 nm. High-resolution MS analyses interfaced with UPLC were done in the Metabolomics unit at the University of Helsinki, Finland as described by Pietiäinen *et al.* (2016).

Accession numbers

The following amino acid sequences were used in phylogenetic analysis: *Vitis vinifera* resveratrol OMT (CAQ76879.1), *Mesembryanthemum crystallinum* PFOMT (AAN61072.1), *Vanilla planifolia* OMT4 (ADZ76153.1), *V. vinifera* anthocyanin OMT (ACO52469.1), *Petunia hybrida* CCoAOMT (ALP75646.1), *P. taeda* CCoAOMT (AAD02050.1), *Rosa chinensis* orcinol OMT (CAD29458.1), *Pinus radiata* D. Don CCoAOMT (ADV40957.1), *Lolium perenne* caffeic acid OMT (AAD10253.1), *Arabidopsis thaliana* CCoAOMT (O49499.1), *A. thaliana* caffeic acid OMT (NP_200227.1), *Medicago sativa* isoflavone OMT (AAC49928.1), *M. sativa* isoliquiritigenin OMT (AAB48059.1), *M. sativa* CCo AOMT (AAC28973.1), *Humulus lupulus* OMT1 (ABZ89565.1), *P. taeda* AEOMT (AAC49708.1) and *A. thaliana* flavone OMT (AAB96879.1).

The enzyme-encoding *P. sylvestris* genes isolated in this work have been deposited in GenBank with the following accession numbers: *PMT1* (KX545298), *PMT2-1* (KX545296), *PMT2-2* (KX858830), *PMT2-3* (KX858831), *PMT2-4* (KX858832), *PMT2-5* (KX858833), *PMT2-6* (KX858834), *PMT2-7* (KX858835), *CCoAOMT* (KX545297) and *AEOMT* (KX545295).

Phylogenetic analysis

Amino acid sequences for different OMTs were aligned with the webPRANK algorithm (Löytynoja & Goldman, 2010). An unrooted tree was constructed by the Maximum Likelihood method with 1000 bootstrap replicates using MEGA 6.06 tool (Tamura *et al.*, 2013). The tree was visualized using the FigTree v.1.4.2. program (http://tree.bio.ed.ac.uk/software/figtree).

Results

Identification and cloning of *PMT2*

Pine transcriptomes were sequenced from mature SW and TZ of 46-yr-old trees, as well as from developing xylem of 5-yr-old seedlings that were wounded by drilling and from needles of 6-wk-old seedlings exposed to UV-C. The transition zone corresponds to a narrow, one to two growth ring wide zone in the wood where the transition of sapwood into heartwood is occurring (Lim *et al.*, 2016). Stilbene production is known to be induced in response to UV-C and wounding stresses, in addition to developmental regulation during heartwood formation (Schoeppner & Kindl, 1979; Bergström, 2003; Harju *et al.*, 2009). Transcripts encoding the Scots pine stilbene synthase (*STS*) were strongly upregulated in these conditions; however, when we analyzed the expression profile of the previously described pinosylvin methylating enzyme-encoding *PMT1* (Chiron *et al.*, 2000a) in our transcriptome libraries, it was either not expressed at all or not coregulated with *STS* (Fig. 2; Table S2). Therefore, we started to screen for a candidate for the true pinosylvin methyltransferase-encoding transcript.

We mapped all reads of our transcriptomic data against the *Pinus* EST collection (The Gene Index Databases, 2014) that consists of assemblies of expressed sequence tags (Tentative Consensus sequences, TCs) generated from 36 pine species (Quackenbush *et al.*, 2000). The reads were mapped also to Trinity assembly of the reads and to the recently published *P. taeda* genome, but mapping against the EST collection worked out the best (Lim *et al.*, 2016). Using mapped read counts we calculated the Pearson correlation coefficient between all TCs and TC154538-encoding Scots pine STS (misannotated, however, as dihydropinosylvin synthase; Fliegmann *et al.*, 1992; Schanz *et al.*, 1992) and extracted putative methyltransferase TCs that had a correlated expression profile. Correlation analysis uncovered as the best candidate TC197639, which was a partial transcript (Table S2). Another fragment TC162768 and one full-length sequence TC166778 (Fig. S2) showed also high correlation. TC197639 and TC162768 turned

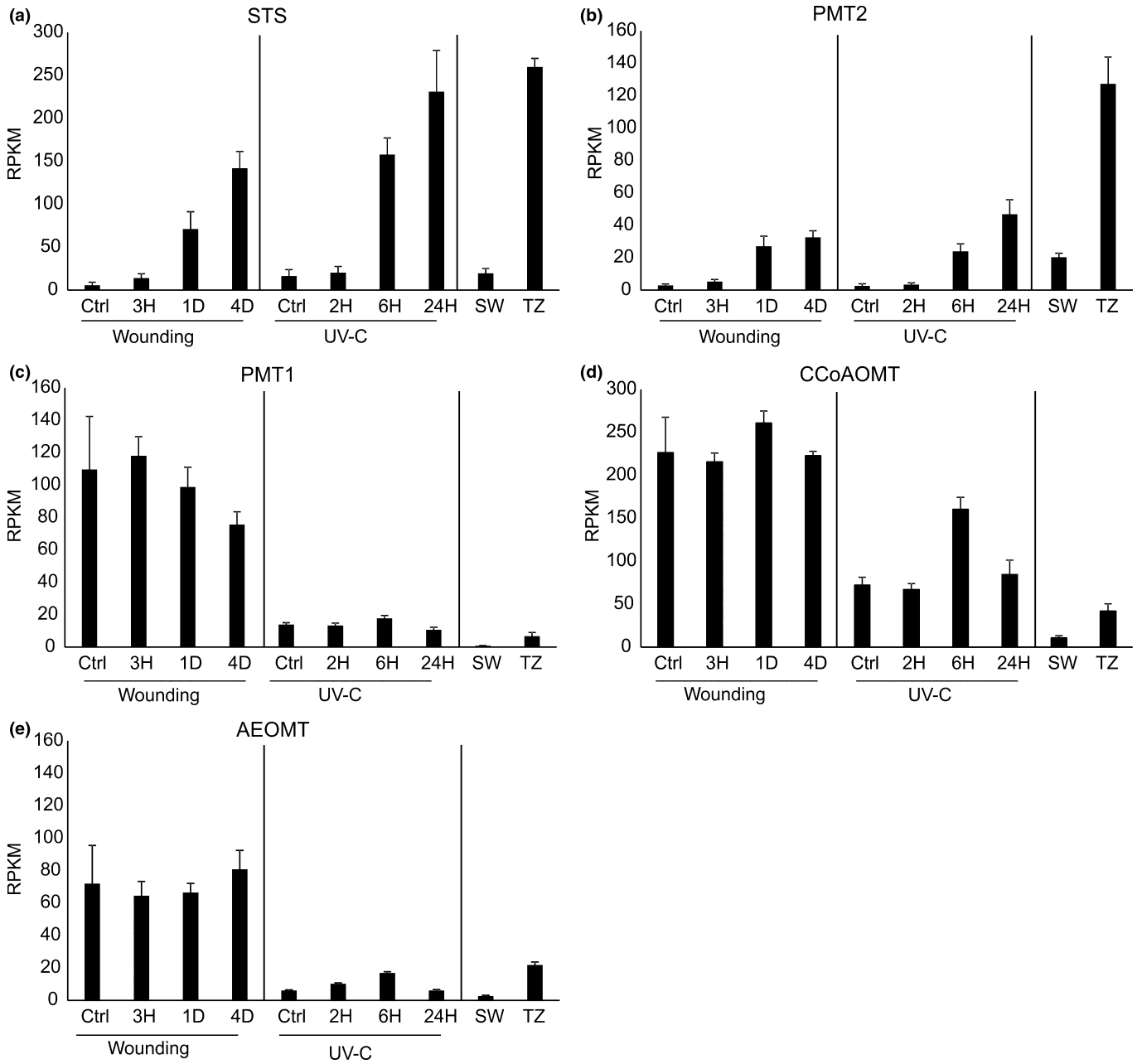

Fig. 2 Expression of (a) *STS*, (b) *PMT2*, (c) *PMT1*, (d) *CCoAOMT* and (e) *AEOMT* in response to wounding of xylem, UV-C exposure of needles and in sapwood (SW) and the transition zone (TZ) in Scots pine. Values are read counts normalized against library size and transcript length (RPKM). Error bars represent the SE of the mean from four replicates. Ctrl, control samples before treatment; *n*H, *n*D, sampling *n* hours or *n* days after the treatment. STS, stilbene synthase; PMT, pinosylvin *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; AEOMT, hydroxycinnamic acids/hydroxycinnamoyl CoA esters OMT; RPKM, normalized read counts.

out to be fragments of TC166778, which we named as *PMT2*. Instead, the TC corresponding to *PMT1* (TC180420) did not show significant correlation with *STS* (Table S2).

The full-length cDNA for *PMT2* was amplified from UV-C, wounding and transition zone samples. We obtained in total seven different clones encoding PMT2, *PMT2-1* to *PMT2-7*. PMT2-1 is characterized in detail in this study. The different variants had nucleotide sequence identity in the coding area between 96% and 99%, and amino acid sequence identity between 94% and 99%, compared with each other (Table S3). All clones from the UV-C-induced needle libraries and some clones from wound-induced xylem libraries had a G-to-T transversion that generated a stop codon after the first in-frame ATG of the cDNA sequence and some of the wounding and TZ clones had a single base deletion between the first and second ATG, causing a frameshift. Presence of these changes in pine mRNA was confirmed by mapping of all the RNA-seq reads against the PMT2-encoding TC (Fig. S3). The alignment of PMT2 amino acid sequence with known methyltransferases as well as the size of a typical type I methyltransferase (*c.* 360 amino acids) suggested

that the first in-frame ATG in the sequence might not be the one where translation is initiated (Louie *et al.*, 2010). Nucleotides in positions −3 and +4 are involved in optimal translational start site recognition (Pisarev *et al.*, 2006) and the second ATG fits better the plant consensus sequence for the initiation of translation (Kozak, 2005). These data are indicative of the second ATG in the transcript being the true initiation site of translation.

The clone *PMT2-1*, amplified from the TZ samples, that had the closest similarity with the TC166778 sequence and did not contain a stop codon or frame shift between the two ATGs was chosen for enzymatic characterization. The open reading frame from the first ATG was 1137 bp, coding for a 41 kDa protein (378 amino acids) and from the second ATG 1086 bp, coding for 39 kDa protein (361 amino acids). Both versions turned out to be active in methylating pinosylvin (see later).

Sequence alignment and phylogenetic analysis of OMTs

Chrystal structure of some plant type I OMTs have been resolved (Zubieta *et al.*, 2001; Louie *et al.*, 2010; Green *et al.*, 2014). Alignment of PMT2-1 amino acid sequence with the perennial ryegrass (*Lolium perenne*) LpCOMT and PMT1 showed that many catalytic residues and residues involved in binding SAM (Louie *et al.*, 2010) of these OMTs are conserved (Fig. 3). From substrate-binding residues, many are shared between PMT1 and LpCOMT yet they are different in PMT2; PMT2 shares only 38% amino acid identity with PMT1.

Phylogenetic analysis shows the clustering of OMTs in two main groups (Fig. 3). Type II OMTs divide into CCoAOMT-like enzymes and true CCoAOMTs responsible for G-type lignin biosynthesis. The Scots pine CCoAOMT characterized in this study falls in the same group with other true CCoAOMTs. AEOMT, PMT1 and PMT2 are type I enzymes. Type I enzymes also divide into two groups. PMT1 clustered together in a group with caffeic acid OMTs, flavone OMT and chalcone OMT. AEOMT and PMT2 are more separated and cluster together with a heterogeneous group of enzymes including, for example, resveratrol and orcinol OMTs.

Gene expression analysis of pine *O*-methyltransferases

Expression of OMT-encoding genes *AEOMT*, *CCoAOMT*, *PMT1* and *PMT2* in UV-C treated needles, wounded xylem and in SW and TZ are shown in Fig. 2. *PMT1* shows strongest expression in the 5-yr-old xylem samples, some expression in the needles and in the TZ and practically no expression SW libraries. Wounding or UV-C treatment had no effect on the expression of *PMT1* (Fig. 2c). Instead, the expression profile of *PMT2* (Fig. 2b) highly resembled the profile of *STS* (Fig. 2a). *PMT2* was expressed at low levels in the control samples and early time points of wounding and UV-C treatment. Expression of the gene was strongly induced in response to both treatments and increased at least until 4 d after the wounding and 24 h after the onset of the UV-C exposure. Furthermore, *PMT2* was highly expressed in the TZ and much less in the SW. *CCoAOMT* (Fig. 2d) was expressed very strongly in the libraries of 5-yr-old seedlings subjected to wounding, but the wounding treatment itself did not affect the expression level of the gene. The gene was also expressed in the UV-C libraries, and stronger in the TZ than mature SW of the tree. UV-C treatment induced the expression

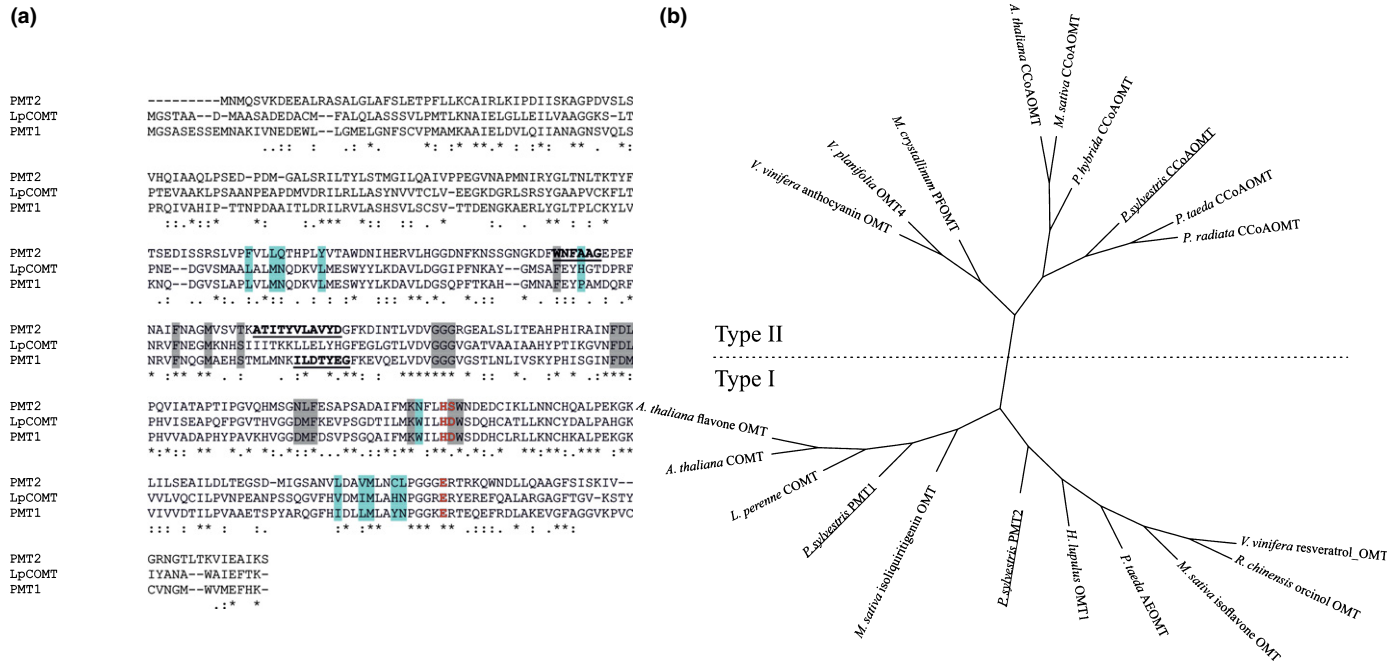

Fig. 3 Amino acid sequence alignment and phylogenetic analysis of *O*-methyltransferases (OMTs). Alignment of pinosylvin *O*-methyltransferase (PMT1 and PMT2) amino acid sequence from Scots pine with *Lolium perenne* LpCOMT (a). *S*-adenosyl-L-methionine (SAM) binding residues are marked in grey, substrate binding residues in blue and residues involved in catalysis in red. In the PMT1 and PMT2 sequences areas underlined are the sequenced amino acid fragments identified by Chiron *et al.* (2000a). '*', fully conserved residue; ':', conservation between groups of strongly similar properties; '.', conservation between groups of weakly similar properties. Unrooted phylogenetic tree of OMTs (b). Accession numbers are listed in the Materials and Methods section.

of *CCoAOMT* which was at its highest level at 6 h after the onset of the treatment. *AEOMT* (Fig. 2e) was expressed strongest in the xylem libraries and to a lesser extent in the UV and TZ libraries. The treatments had no effect on the transcription and the gene was only weakly expressed in the SW. Expression of OMT-encoding genes in samples used to generate SOLiD sequencing libraries were confirmed with RT-qPCR. Expression profiles of transcripts in RT-qPCR analysis from pooled samples resembled closely the expression profile obtained from SOLiD sequencing although expression levels of *PMT1* in xylem seemed to be relatively lower in this assay (Fig. S4).

Analysis of PMT2 variants

Relatively high sequence variation between PMT2 clones suggests a possibility that some of them might derive from paralogous genes instead of alleles. With a threshold of 98% sequence identity in nucleotides, *PMT2-2* and *PMT2-3*, as well as *PMT2-4*, *PMT2-5* and *PMT2-6*, might represent allelic forms, but identities between other pairs never reaches 97% (Table S3). This grouping would indicate presence of four paralogous PMT2 loci. To address further the question if PMT2 is encoded by multiple genes in the genome, we amplified and sequenced a genomic fragment of the gene that contains two introns. The fragment was amplified from DNA obtained from the haploid megagametophyte tissue of four separate seeds of a single Scots pine mother tree. Four clones from each DNA sample were sequenced. Sequencing revealed six different kind of introns, which indicates that there are at least three PMT2-encoding genes in the genome. Also the loblolly pine genome seems to have three to four copies of the *PMT2* gene. This was analyzed by searching *PMT2* homologous sequences from the loblolly pine genome, aligning them with the *PMT2* cDNA sequence and inspecting the intron sequences retrieved this way.

Addressing the question if different paralogs or alleles are preferentially expressed in different tissues or stress conditions, is difficult with short RNA-seq reads because individual single nucleotide polymorphisms (SNPs) are sparse in the sequence and often shared with different versions. There is, however, some sequence variation in the 3′ UTR, especially in *PMT2-1* and *PMT2-7*. Mapping all the reads to the short 123–145 bp fragment from the 3′ UTR with high stringency showed that most of the variants are induced in the TZ and wounding libraries, with the exception that *PMT2-7* has low expression in the TZ. *PMT2-7* is preferentially expressed under UV-C and wound induction, *PMT2-6* under wound induction and in TZ, and *PMT2-1* in the TZ. Investigating the patterns of SNPs in the coding sequences shows a similar pattern, but the resolution of this analysis is not very high.

Substrate specificities of OMTs and *in vitro* kinetic parameters for the recombinant enzymes

PMT1, PMT2-1, AEOMT and CCoAOMT were expressed in *E. coli* as N-terminally His-tagged recombinant proteins. Most experiments for PMT2 were done with the shorter version of the enzyme but also the longer version is active and has similar kinetic parameters with PS as substrate. Methylation activities of the purified proteins were screened against 12 different substrates from different secondary metabolite groups using [^{14}C]-SAM as a methyl group donor. A bacterial lysate transformed with empty vector was used as a control. The tested stilbene structures included PS, resveratrol, piceatannol and PSME that differ from each other at the level of hydroxylation and methylation. From flavonoids, the flavonols kaempferol and quercetin, flavones luteolin and apigenin, the flavanone naringenin and the flavanonol dihydroquecetin (DHQ) were tested. In addition, the hydroxycinnamic acids 5-OH ferulic acid and caffeic acid, and its CoA ester caffeoyl-CoA, were tested as substrate (Fig. 4).

PMT2 was shown to be an enzyme that specifically methylates only stilbenes. Activity of the enzyme decreases with increasing number of hydroxyl groups in the molecule (Table 1; Fig. 4). PS (3,5-dihydroxystilbene) was methylated efficiently, resveratrol (3,5,4′-trihydroxystilbene) to a lesser extent and piceatannol (3′,4′,3,5-tetrahydroxystilbene) only weakly. PMT1 and CCoAOMT instead turned out to be multifunctional enzymes capable of methylating several substrates from different substrate groups *in vitro* (Table 1; Fig. 4). PMT1 most strongly methylated piceatannol, caffeic acid and quercetin; it also was able to methylate the hydroxyl group in the *meta* position of stilbenes PS and resveratrol but not as strongly as in picetannol, caffeic acid or quercetin. Caffeoyl-CoA was methylated but to a much lesser extent than the corresponding acid. PMT1 had only a weak activity against 5-OH ferulic acid and PSME. Kaempferol, naringenin, apigenin and DHQ were not methylated. The substrate specificity of CCoAOMT was strictly dependent on the vicinal hydroxyl groups at 3′ and 4′ positions of the molecule (Kopycki *et al.*, 2008). Caffeoyl-CoA, luteolin, quercetin and piceatannol were all efficiently methylated (Table 1; Fig. 4). In contrast to PMT1, CCoAOMT favored caffeoyl-CoA instead of caffeic acid, was able to methylate DHQ and had higher activity towards 5-OH ferulic acid than PMT1. CCoAOMT had no activity against PS, PSME, resveratrol, kaempferol, apigenin and naringenin that are lacking the vicinal hydroxyl groups. Bacterial lysate control containing empty expression vector and the purified AEOMT did not show any activity against any of the tested substrates.

Kinetic parameters of PMT1 and PMT2 were measured with PS as the substrate (Table 2). We defined the initial reaction velocities against varying substrate concentration (Fig. 5). The K_m values for the enzymes were 16 μM for PMT1 and 44 μM for PMT2. However, the V_{max} of PMT2, 9.9 μkat mg $^{-1}$, was more than triple that of PMT1, 2.7 μkat mg^{-1}, and thus the V_{max} : K_m ratio indicating catalytic efficiency of PMT2 was higher than that of PMT1 (Table 2). Kinetic parameters also were measured for the longer version of PMT2 and were very similar to those of the shorter version (K_m 31 μM and V_{max} 8.8 μkat mg^{-1}).

Verification of methylated products by HPLC and UPLC-MS/MS

The *in vitro* enzymatic assays showed that PMT2 was able to methylate PS. The identity of the methylated product was determined using HPLC and UPLC-MS/MS. The retention time of the product in HPLC was compared with the retention time of

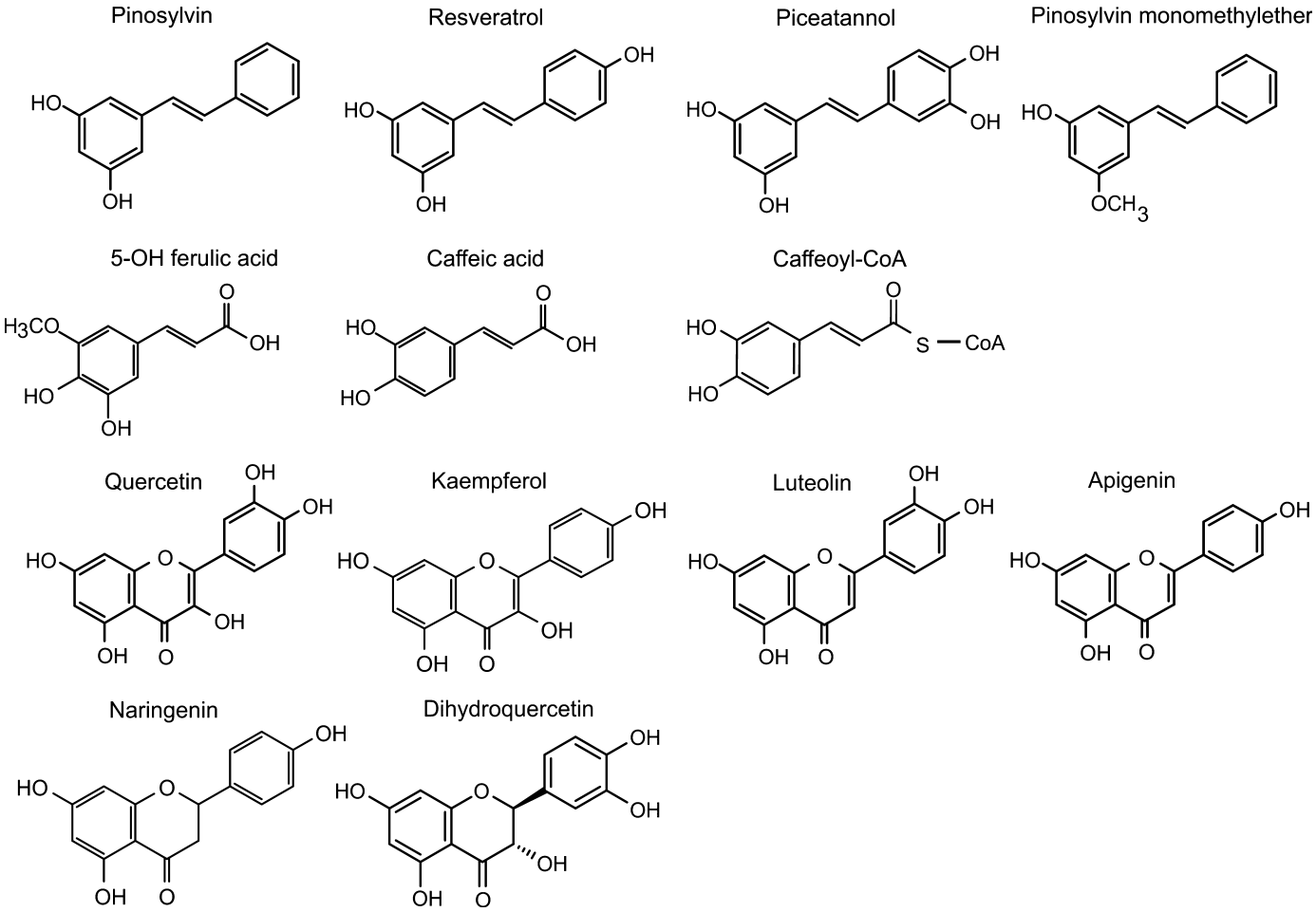

Fig. 4 Structures of the compounds used in screening of methylation activities of the enzymes.

Table 1 Methylation activities of pinosylvin *O*-methyltransferase (PMT1 and PMT2) and caffeoyl-CoA *O*-methyltransferase (CCoAOMT) against a variety of substrates

Substrate	Substrate group	PMT1[a]	PMT2[a]	CCoAOMT[b]
Pinosylvin	Stilbene	100	100	–
Resveratrol	Stilbene	59	33	–
Piceatannol	Stilbene	236	8	122
Pinosylvin monomethylether	Stilbene	< 2	–	–
5-OH ferulic acid	Hydroxycinnamic acid	14	–	10
Caffeic acid	Hydroxycinnamic acid	209	–	2
Caffeoyl-CoA	CoA thioester	28	–	100
Kaempferol	Flavonol	–	–	–
Quercetin	Flavonol	191	–	124
Luteolin	Flavone	90	–	124
Apigenin	Flavone	–	–	–
Naringenin	Flavanone	–	–	–
Dihydroquercetin	Flavanonol	–	–	19

–, no detectable activity.
[a]Relative activities (%) compared with pinosylvin.
[b]Relative activities (%) compared with caffeoyl-CoA.

Table 2 Kinetic parameters of pinosylvin *O*-methyltransferase (PMT1 and PMT2) with pinosylvin

	K_m (μM)	V_{max} (μkat mg^{-1})	V_{max}/K_m
PMT1	16.1 ± 5	2.7 ± 0.2	0.17
PMT2 (short)	43.7 ± 4.6	9.9 ± 0.4	0.23
PMT2 (long)	31.7 ± 3.6	8.8 ± 0.3	0.28

Values represent mean ± SE.

commercial standard and shown to be identical to that of PSME. The fragmentation pattern of the product compared with that of PSME further confirmed that the product formed in the reaction was 3-methoxylated (Fig. 6).

For the other enzymatic reactions, some of the methylated products were characterized using either HPLC, UPLC-MS/MS or both (Table S4). Both PMT1 and CCoAOMT were able to methylate quercetin to isorhamnetin and CCoAOMT caffeoyl-CoA to feruloyl-CoA, based on spectral data from high performance liquid chromatography–diode-array detection (HPLC-DAD).

Both PMT1 and CCoAOMT methylated piceatannol to a product that had identical retention time in HPLC and

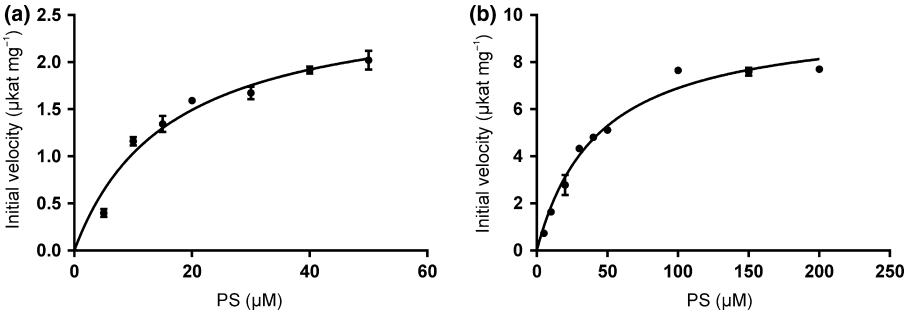

Fig. 5 Initial velocity of the (a) PMT1 and (b) PMT2 enzymatic reactions as the function of concentration of pinosylvin (PS). Error bars represent ± SE. PMT, pinosylvin *O*-methyltransferase.

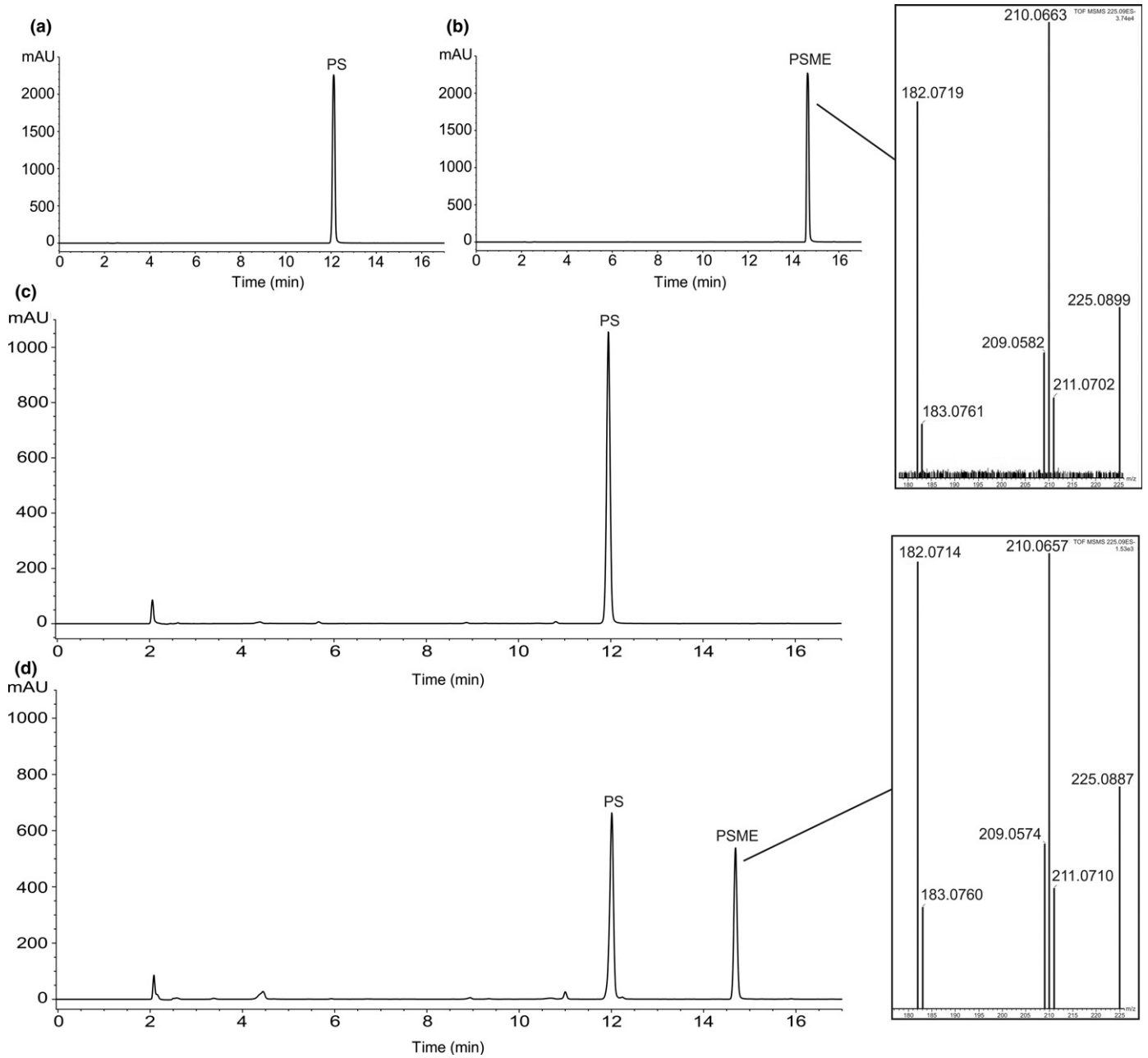

Fig. 6 high performance liquid chromatography–diode-array detection (HPLC-DAD) and ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis of the *in vitro* methylation product of pinosylvin (PS) by PMT2. HPLC analysis of commercial standards of pinosylvin (3,5-dihydroxystilbene, PS) and its monomethylether (3-methoxy-5-hydroxystilbene, PSME) are shown in (a) and (b), respectively. Enzymatic assay with bacterial lysate transformed with empty vector (c) and enzymatic assay with PMT2 (d). Peaks are given in milli Absorbance Units (mAU) at 276 nm.

fragmentation pattern in MS/MS. The commercial standard for the predicted product isorhapontigenin was not available. According to literature, the fragmentation of isorhapontigenin and rhapontigenin in MS/MS are identical leading the main daughter ions of 241 and 224 (Fernández-Marín *et al.*, 2012). For rhapontigenin, a commercial standard was available and it fragmented as the product formed in enzymatic reactions (Table S4). In HPLC the retention time of rhapontigenin and isorhapontigenin are slightly different (Setoguchi *et al.*, 2014). The commercial standard had slightly different retention time compared with product formed in enzymatic reactions (presumably isorhapontigenin). However, because CCoAOMT strictly methylates the *meta* position in the structure (Kopycki *et al.*, 2008), we have no reason to believe that the formed product would be other than isorhapontigenin for both enzymes.

Discussion

We have isolated a new selective *O*-methyltransferase, PMT2 that preferentially methylates pinosylvin (3,5-dihydroxystilbene, PS) into its monomethylether (3-methoxy-5-hydroxystilbene, PSME) *in vitro* and is specific to stilbenes as substrate. Expression analysis supports the role of PMT2 in stilbene biosynthesis during heartwood formation and in response to UV-C and wounding stresses, and suggests that it is the true *O*-methyltransferase (OMT) for the stilbene pathway in pine, instead of the previously characterized PMT1 (Chiron *et al.*, 2000a).

PMT2 seems to be encoded by small gene family including at least three members. Stilbene synthase (STS) is also encoded by gene family of four members sharing amino acid identity of > 95% (Preisig-Müller *et al.*, 1999).

PMT2 is coexpressed with STS in all experimental conditions tested, suggesting that they are under common regulation. PMT1, by contrast, was expressed highest in the stems of the 5-yr-old seedling samples that consisted mostly of developing xylem tissue, and none of the tested treatments had any effect on its expression. A previous study did show induction of *PMT1* gene expression in needles in response to ozone, and in phloem in response to fungal treatment (Chiron *et al.*, 2000b). PMT1 thus might have a role in methylating PS or some other metabolite during ozone stress and in response to fungal attack. However, weak gene expression in the transition zone indicates that PMT1 is not involved in methylating PS in the developmental context of heartwood formation. Furthermore, it is not induced in needles by UV-C or in the xylem by wounding.

PMT2 is a selective enzyme methylating only PS, resveratrol and piceatannol. Resveratrol or piceatannol are not produced naturally in pine and the enzyme activity towards both of them can be explained by structural similarity of these compounds with PS (Fig. 4). PMT1, conversely, is a multifunctional enzyme able to use several different compounds from flavonoids to hydroxycinnamic acids as substrate and it seems to prefer vicinal hydroxyl groups.

In their work leading to isolation of PMT1, Chiron *et al.* (2000a) elicited Scots pine callus cultures with the fungal pathogen *Lophodermium seditiosum*. As a response to pathogen treatment, PS methylating activity of the callus cultures increased about two-fold. Active fractions from the induced cultures were collected and separated in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) resulting in two protein spots of size 37 and 40 kDa. Proteins were tryptically digested and sequenced resulting in three peptide sequences. One of the peptide sequences (ILDTYEG), originating from the 40 kDa protein was part of PMT1, a cDNA molecule amplified from ozone treated needles using a primer for conserved region in plant OMTs. The two other sequences (ATITDVLAVYD and V?NFAAG), from the 37 kDa protein, were not present in the deduced amino acid sequence of PMT1. In fact, both of these peptides are part of the amino acid sequence of PMT2. Apparently PMT2 was copurified with PMT1 in Chiron's study and for that reason, the enzymatic assays with the active fraction show combined activities of both PMT1 and PMT2. The purified fraction showed Michaelis–Menten kinetics towards PS with a K_m value of 49 μM, which is in the same range with 44 μM measured for the PMT2 recombinant enzyme in this study. For the recombinant PMT1, K_m was smaller: 14 μM in Chiron's study and 16 μM in our experimental system. All of these values are in the same range of 10–70 μM as measured for other plant OMTs (Nagel *et al.*, 2008; Schmidlin *et al.*, 2008; Hugueney *et al.*, 2009). The catalytic efficiency of PMT2 towards PS was, however, higher than that of PMT1.

Caffeoyl-CoA *O*-methyltransferase (CCoAOMT) has been shown to be involved in biosynthesis of G-type lignin (Li *et al.*, 1999; Wagner *et al.*, 2011). Strong reduction of *CCoAOMT* expression in transgenic *P. radiata* tracheary element cultures resulted in a moderate (5–20%) of reduction in lignin content and in a five- to 10-fold higher H : G ratio (Wagner *et al.*, 2011). Thus, it was speculated that there might be other methyltransferases capable of methylating caffeoyl-CoA. PMT1 is expressed strongest in developing xylem and it is shown to have even higher specific activity towards caffeoyl-CoA than towards PS (Chiron *et al.*, 2000a). PMT1 might then be one of the methyltransferases preventing greater reductions of lignin content in transgenic *P. radiata* when *CCoAOMT* is downregulated.

CCoAOMT belongs to the class II OMTs that are composed of true CCoAOMTs involved mostly in lignin biosynthesis and of CCoAOMT-like enzymes involved in biosynthesis of other secondary metabolites like flavonoids and not showing strong methylation activity towards caffeoyl-CoA (Hugueney *et al.*, 2009; Widiez *et al.*, 2011). CCoAOMT in the present study is very similar to the lignin-forming CCoAOMT, being 98% identical to *P. radiata* CCoAOMT, and is then considered to be a true CCoAOMT (Wagner *et al.*, 2011). In general, CCoAOMTs are strictly specific for the *meta* position of aromatic vicinal hydroxyl groups (Kopycki *et al.*, 2008). CCoAOMT was shown here to strongly methylate luteolin, quercetin and piceatannol, in addition to caffeoyl-CoA. It showed very similar substrate preference to PaCCoAOMT isolated from Norway spruce (*Picea abies*) that methylated caffeoyl-CoA, luteolin and quercetin, and did not use kaempferol and naringenin as substrates (Kim *et al.*, 2010). We observed that *CCoAOMT* shows considerably high expression in needles although they are not strongly lignified. UV-C treatment further induced the expression of *CCoAOMT*.

We propose that either the reactive oxygen species (ROS) burst caused by UV-C is able to induce lignin biosynthesis (Barros *et al.*, 2015) or that CCoAOMT has other roles in pine in addition to methylation of caffeoyl-CoA for G-type lignin formation. One possibility is that CCoAOMT could be involved in methylation of quercetin into isorhamnetin also *in vivo*. Both quercetin and isorhamnetin 3-*O*-glucosides, together with kaempferol glucosides, are the major flavonol types accumulating in pine needles, and production of diacylated derivative of isorhamnetin have shown to increase in response to UV-B exposure (Lavola *et al.*, 2003; Kaffarnik *et al.*, 2005). In Petunia, PhCCoAOMT1 has a dual role in production of phenylpropenes eugenol and lignin (Shaipulah *et al.*, 2016), and in *Arabidopsis thaliana* downregulation of CCoAOMT reduces the amounts of isorhamnetin in addition to lignin (Do *et al.*, 2007).

We showed that CCoAOMT efficiently methylates piceatannol *in vitro*, forming isorhapontigenin. In Norway spruce, the glucosylated form of isorhapontigenin, isorhaphontin, is the main stilbene together with astringin. They are synthesized from resveratrol, formed by a stilbene synthase, but the enzymes making further modifications are unknown (Hammerbacher *et al.*, 2011). Spruce CCoAOMT shares 95% identity at amino acid level with the pine CCoAOMT, so it is possible that CCoAOMT is the enzyme responsible of methylating piceatannol in the stilbene pathway of spruce.

In this study, we showed that PMT2 is the selective enzyme involved in methylation of PS into its monomethylether and based on its expression profiles we suggest that it is the OMT responsible for both developmentally and stress induced PSME biosynthesis. The previous candidate for PS methylation, PMT1, as well as CCoAOMT are multifunctional enzymes. According to their expression profiles and enzymatic activities, PMT1 is likely to be involved in some other biosynthetic pathway than the stilbene pathway, and CCoAOMT may have functions in stilbene or flavonoid pathways in addition to lignin biosynthesis.

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Author contributions

T.P. designed and performed the research, analyzed and interpreted the data and wrote the manuscript; K-J.L. constructed the RNA sequencing libraries, performed the bioinformatics analysis and did the RT-qPCR analysis; M.P. participated in designing the research; and T.H.T. designed the research, interpreted the data and wrote the manuscript with T.P.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Purified His tagged recombinant proteins.

Fig. S2 Tentative consensus sequence coding for PMT2 from *Pinus* EST collection, The Gene Index Databases 2014.

Fig. S3 Alignment of RNA-seq reads from UV-C, wounding and TZ/SW libraries against TC166778 coding for PMT2.

Fig. S4 RT-qPCR analysis of STS, PMT2, PMT1 and CCoAOMT-encoding genes in response to wounding of xylem, UV-C exposure of needles and in SW and TZ.

Table S1 Primers used in this study

Table S2 Correlation analysis of STS and putative *O*-methyltransferase-encoding TCs across transcriptomic libraries

Table S3 Percentage identity matrices created by CLUSTAL v.2.1 for different versions of PMT2

Table S4 Retention times in HPLC and UPLC-MS or UPLC-MS/MS analysis of *in vitro* reaction products

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