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Caffeic Acid O-Methyltransferase from *Populus deltoides*: Functional Expression and Characterization

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Enzymatic O-methylation, catalyzed by S-adenosyl-L-methionine (SAM)-dependent O-methyltranferases (OMTs), is a ubiquitous reaction, occurring in almost all living organisms. Plant OMTs are involved in the methylation of secondary metabolites, including phenylpropanoid and flavonoid compounds. Here, we used RT-PCR to isolate and characterize *POMT-2* from *Populus deltoides*. This OMT comprises a 1095-b open reading frame that encodes a 39.7-kDa protein. BLAST results showed 87% identities to an OMT from *Prunus dulcis* and a caffeic acid OMT from *Rosa chinensis*. POMT-2 was expressed in *Escherichia coli* as a glutathione S-transferase fusion protein, and was purified by affinity chromatography. POMT-2 transferred a methyl group of SAM to caffeic acid and 6,7-dihydroxyflavone, but showed low activities toward quercetin and kaempferol. According to its *in vitro* substrate preference and composition of phenolic compounds in poplar, the *in vivo* function of POMT-2 is probably the methylation of caffeic acid and an involvement in lignin biosynthesis.

Keywords: caffeic acid, flavonoids, O-methyltransferase

Lignin, a polymer of aromatic subunits, is derived from phenylalanine via the phenylpropanoid pathway. Its basic subunits are hydroxylated and methoxylated phenylpropanoid units, i.e., monolignols, para-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Whetten et al., 1998). In dicots, these are primarily monomethylated guaiacyl (G) units derived from coniferyl alcohol as well as dimethylated syringyl (S) units from sinapyl alcohol (Neish, 1968). Trees need large amounts of lignin, i.e., 15 to 36% of total wood dry weight, for both structural support and the transport of nutritive elements.

Coniferyl alcohol and sinapyl alcohol undergo important reactions, i.e., hydroxylation and O-methylation, during the biosynthesis of monolignol (Neish, 1968; Chapple, 1998; Yang et al., 2005). O-Methylation, mediated by O-methyltransferases (OMTs), is ubiquitous, occurring in almost all organisms, including bacteria, fungi, plants, and mammals. A large number of plant OMTs are involved in the secondary metabolism of products such as flavonoids, alkaloids, coumarins, and lignins (Ibrahim et al., 1998).

OMTs modulate physiological processes, accounting for the structural differences and properties of lignin. Both caffeic acid O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCoA-OMT) are involved in this lignin biosynthesis. COMT, ranging in size from 38 to 43 kDa, methylates caffeic acid and 5-hydroxyferulic acid. Initially, COMT that used those two acids as substrates during monolignol biosynthesis was thought to be a bifunctional enzyme (Inoue et al., 2000). CCoA-OMT, at 26 to 28 kDa, utilizes caffeoyl-CoA or 5-hydroxylferuloyl-CoA as a substrate (Ye et al., 2001). These two OMTs represent distinct groups involved in lignin biosynthesis. Although genome projects with various species have deposited a myriad of OMT gene sequences in databases, most of their substrates have not been determined. This step, however, is prerequisite to exploring the *in vivo* functioning of individual OMT genes. Here, we report the molecular and biochemical characterization of a poplar OMT, *POMT-2*, that encodes a COMT.

MATERIALS AND METHODS

Chemicals

The following are the sources for our research chemicals: flavonoids, from Indofinechemicals (USA); other phenolic compounds, from Sigma (USA); and HPLC-Grade liquid solvents, from Merck (Germany).

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Cloning of *POMT-2*

Total RNA from poplar (*Populus deltoides*) leaves was isolated with a plant total RNA isolation kit (USA). The cDNA was synthesized as described by Kim et al. (2003). For PCR we used Hot start Taq DNA polymerase (Qiagen) under the following conditions: 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min amplification at 72°C. *POMT-2* primers were designed based on the sequence from The Institute of Genome Research (TIGR; accession number TC20213): forward primer, 5'-CGATCAAGATGGGTTCAACA-3' and reverse primer, 5'-TTAAGCCTTAGGCCTGCTTG-3' (translation initiation codon and translation stop codon are underlined). The PCR product was subcloned into a pGEMT-easy vector (Promega, USA) and the resulting plasmid was sequenced.

Expression and Induction of POMT-2 in *Escherichia coli*

The open reading frame of *POMT-2* was subcloned into pGEX 5X-1 (Amersham, USA), and transformants were grown in an LB medium containing 50 μg mL^{-1} ampicillin. The cultures grew until their absorbance at 600 nm reached 0.6. At that point, IPTG was added to a final concentration of 0.1 mM, and the transformants were grown for 5 h at 30°C. The induced POMT-2 was purified on a Sepharose 4B column (Amersham).

Enzyme Assay and Analysis of Metabolite

To measure *O*-methyltransferase activity, we used a reaction mixture containing 50 μg of the purified recombinant protein, 2 mM DTT, 40 μM SAM, and 100 μM substrate in 10 mM Tris/HCl buffer (pH 7.5), at a final volume of 500 μL. This mixture was incubated at 37°C for 30 min and extracted twice with ethyl acetate, before the organic layer was evaporated to dryness. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were performed as described by Park et al. (2003).

Nuclear Magnetic Resonance Spectroscopy

To identify the reaction product of the 6,7-dihydroxyflavone produced by POMT-2, nuclear magnetic resonance (NMR) experiments were conducted as described by Kim DH et al. (2005). An eluent containing the product was collected 20 times on HPLC and evaporated under reduced pressure. The dried remnant was then dissolved in ethylacetate, and the supernatant was separated by centrifuge and evaporated again under reduced pressure. The final remnant was dissolved in dimethylsulfoxide-d6 for the NMR experiments.

RESULTS AND DISCUSSION

Cloning of *POMT-2*

Our *POMT-2* gene sequence, retrieved from the TIGR poplar gene sequencing project, was cloned using RT-PCR. The resulting PCR product was then subcloned and sequenced. Its open reading frame consisted of 1095 b encoding a 39.7-kDa protein. BLAST results showed that POMT-2 shares 87% identities with both the OMT from *Prunus dulcis* (GenBank accession number 602588) and the caffeic acid OMT from *Rosa chinensis* (GenBank accession number 27527920).

Because *O*-methyltransferases utilize SAM as the methyl group donor, the SAM binding sites in OMTs are well-conserved (Kim BG et al., 2005). For instance, we found that eight amino acid residues involved in the AdoMet binding site, including chalcone *O*-methyltransferase (ChOMT), isoflavone *O*-methyltransferase (IOMT), and caffeic acid *O*-methyltransferase (COMT), were similar, although these enzymes utilize different substrates (Zubieta et al., 2001, 2002). That POMT-2 SAM binding site also was predicted by comparing the POMT-2 amino acid sequence with other OMTs. This POMT-2 SAM binding site was the same as in those three other OMTs (Fig. 1). We were also able to predict the substrate binding site at which the eight amino acids are involved. POMT-2 has the same binding site as COMT; predicting the substrate binding sites strongly suggests that POMT-2 encodes a COMT. However, we cannot exclude the possibility that a region other than the substrate binding site could contribute to such substrate discrimination. For example, two *O*-methyltransferases -- OMTII-1 and OMTII-4 -- of meadow rue (*Thalictrum tuberosum*), which exhibit high sequence identity, differ by only one amino acid in their sequence of 364 amino acids, whereas the binding affinity for caffeic acid shows a 4-fold difference (Frick and Kutchan, 1999) even though the different residue is located at a position that is not part of the substrate binding site.

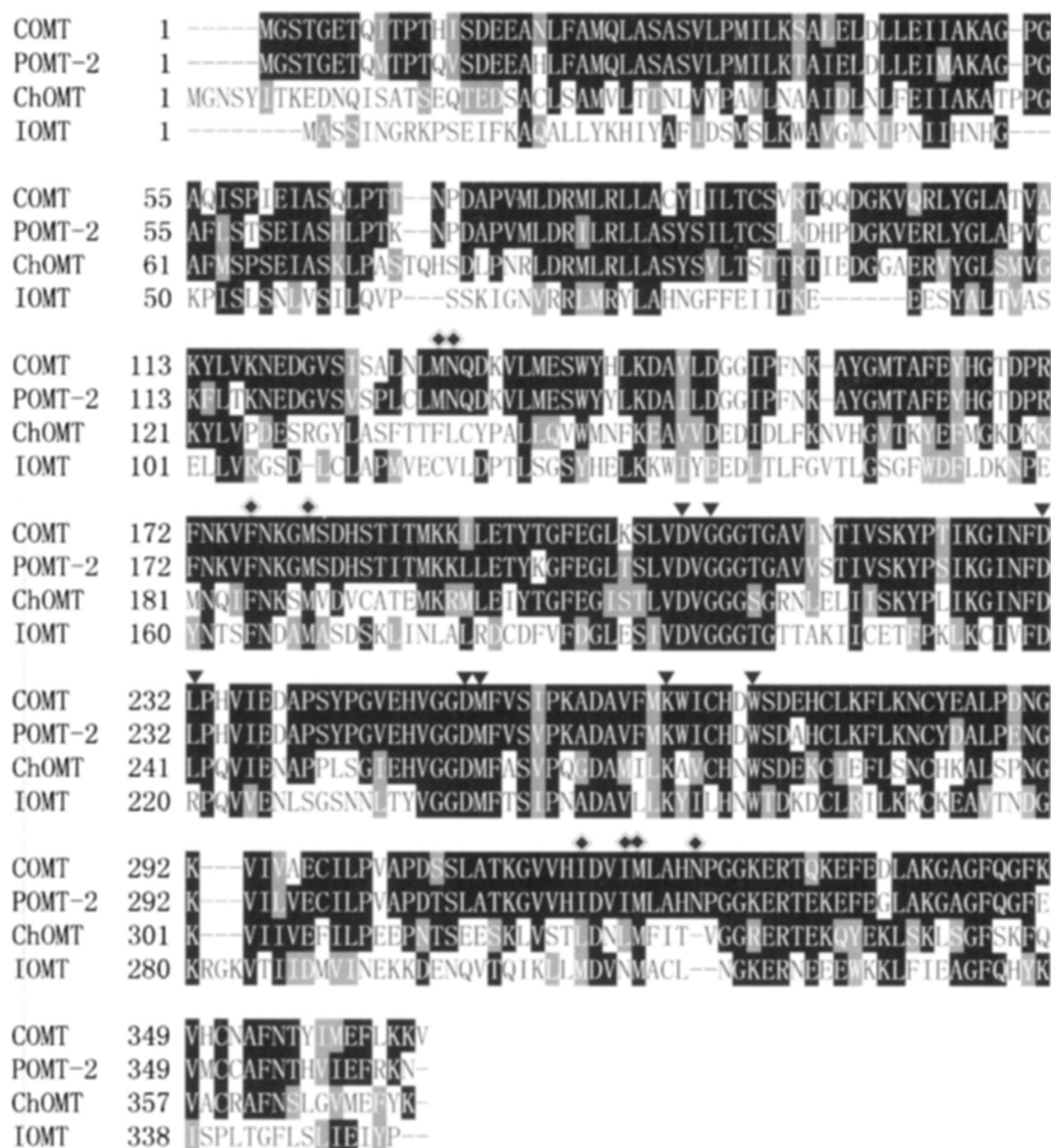

Figure 1. Amino acid sequence alignments of POMT-2 with four representative plant *O*-methyltransferases. residues involved in SAM binding are indicated by ▼ at top of amino acid; residues neighboring substrate are indicated by ◆ at top of amino acid. COMT: Caffeic acid *O*-methyltransferase from *Medicago sativa* (GenBank Accession number 166420). POMT-2: Caffeic acid *O*-methyltransferase from *P. deltoides*. IOMT-2: Isoflavone *O*-methyltransferase from *M. sativa* (GenBank Accession number 2580584). ChOMT: Chalcone *O*-methyltransferase from *M. sativa* (GenBank Accession number 1843462).

Substrate Determinations for POMT-2

To evaluate our substrates, we expressed *POMT-2* in *E. coli* as a glutathione *S*-transferase (GST) fusion protein, then purified this expressed recombinant protein to near homogeneity, as indicated by SDS-PAGE (Fig. 2). The molecular weight of the recombinant POMT-2 was about 65.7 kDa, which agrees with the sum of the predicted molecular weight of POMT-2 plus GST (26.5 kDa).

BLAST results showed that POMT-2 did mediate *O*-methylation reactions. Therefore, to determine their suitability as substrates with purified recombinant POMT-2, we tested several phenolic compounds including caffeic acid, *p*-coumaric acid, orcinol, esculetin, apigenin, 6,7-dihydroxyflavone, eriodictyol, luteolin, kaempferol, and quercetin. The reaction products were first analyzed by TLC. *p*-Coumaric acid, orcinol, apigenin, and kaempferol did not give any new product while the others generated a new product

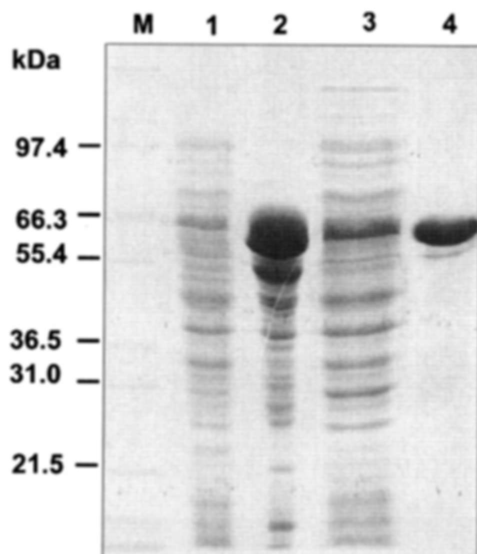

Figure 2. Expression and purification of recombinant POMT-2. M, Molecular weight marker; 1, Uninduced *E. coli* lysate; 2, Induced *E. coli* total lysate; 3, Soluble fraction of induced *E. coli* lysate; 4, Purified recombinant POMT-2.

that had a different R_f value from the substrates. Those compounds that did undergo a reaction contained orthohydroxyl groups whereas the others did not. The reaction products from caffeic acid, esculetin, 6,7-dihydroxyflavone, eriodictyol, luteolin, and quercetin were then analyzed further with HPLC. Retention times were the same for the reaction product of caffeic acid (Fig. 3B) as for that of ferulic acid, i.e., the 3′-methoxy caffeic acid (Fig. 3C). Furthermore, 6,7-dihydroxyflavone produced a peak at 13.8 min (Fig. 3D) while the reaction product generated a new peak at 15.8 min (Fig. 3E). Because an authentic sample of the reaction product for 6,7-dihydroxyflavone was not commercially available, we identified it via NMR spectroscopy. The new ^{13}C peak at 56.3 ppm was not observed in the ^{13}C NMR spectrum of the reactant, although 6,7-dihydroxyflavone was shown in the ^{13}C NMR data of the product. Therefore, it was expected that this product contained a methoxy group. We also compared the ^{1}H and ^{13}C NMR data of the reaction product (Table 1) with those of 6,7-dihydroxyflavone, whose data (not shown) were determined in our lab. This comparison revealed only one difference between those two compounds, i.e., the existence of a methyl group. To locate the methylated position, we performed nuclear overhauser exchanged spectroscopy (NOESY), with a mixing time of 1 s. Because an nOe cross peak between H-8 at 7.34 and the methyl proton at 3.94 ppm was observed in NOESY, the methylated position was determined as 7-OH.

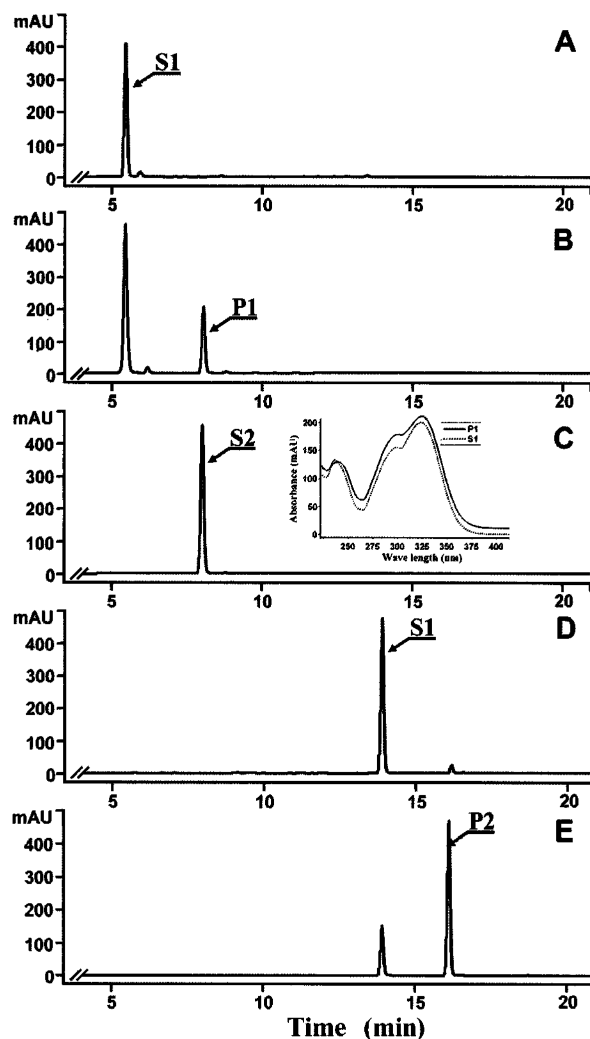

Figure 3. HPLC profile of reaction products with POMT-2. **A,** Authentic caffeic acid; **B,** Caffeic acid reaction product; **C,** Authentic ferulic acid; **D,** Authentic 6,7-dihydroxyflavone; **E,** 6,7-Dihydroxyflavone reaction product. Right inset in **C** is UV spectra for authentic ferulic acid (S1) and caffeic acid reaction product (P1).

Substrate preference by POMT-2 was examined by analyzing the amount of reaction product with the peak area from HPLC analysis. Among the substrates tested, the best was 6,7-dihydroxyflavone, followed by caffeic acid and esculetin (Table 2). The flavonoids quercetin, eriodictyol, and luteolin all showed less than 20% utility compared with 6,7-dihydroxyflavone. Kinetic parameters for 6,7-dihydroxyflavone and caffeic acid were also determined through Lineweaver-Burk blots (Table 2). POMT-2 exhibited a higher affinity for the former than for the latter, as

Table 1. Assignments of ^{1}H and ^{13}C data for the reaction product produced by POMT-2 and the substrate 6,7-dihydroxyflavone.

Position	δ of ^{1}H ppm^{-1}		δ of ^{13}C ppm^{-1}	
	Reaction product	6,7-Dihydroxyflavone	Reaction product	6,7-Dihydroxyflavone
3	6.89 (s)	6.83 (s)	106.1	106.0
5	7.31 (s)	7.31 (s)	107.2	107.6
8	7.34 (s)	7.04 (s)	100.7	100.3
2'/6'	8.07 (m)	8.02 (m)	126.0	126.0
3'/5'	7.57 (m)	7.54 (m)	131.4	129.1
4'	7.58 (m)	7.55 (m)	129.1	131.3
7-OMe	3.94 (s)	–	56.3	–

s, singlet; m, multiplet.

Table 2. Substrate specificity of POMT-2.

Substrate	Relative activity (%)	K_m value (mM)	V_{max} (pkat mg^{-1})	V_{max}/K_m	K_{cat}/K_m (mM^{-1} s^{-1})
6,7-dihydroxyflavone	100	65.1	256.4	3.9	0.077
Caffeic acid	88	74.7	158.7	2.1	0.029
Esculetin	63	ND	ND	ND	ND
Quercetin	19	ND	ND	ND	ND
Luteolin	19	ND	ND	ND	ND
Eriodictyol	7	ND	ND	ND	ND

Enzyme assays were carried out using 50-80 μg of purified recombinant protein, 10-150 μM substrate, and 80 μM of *S*-adenosyl methionine. Incubation was at 37°C for 30 min.

indicated by low K_m values and high V_{max}/K_m ratios (Table 2). Based on that substrate affinity (K_m value) and K_{cat}/K_m value, we identified 6,7-dihydroxyflavone as the best substrate. Other OMTs also use not only caffeic acid but also a flavonoid. For example, OMT1 and OMT2 from *Chrysosplenium americanum* catalyze 3′-*O*-methylation of the flavonoids luteolin and quercetin, although they also catalyze the efficient 3-*O*-methylation of the phenylpropanoid caffeic acid (Gauthier et al., 1998). Their *in vivo* function is predicted to mediate flavonoid *O*-methylation because *C. americanum* contains highly methylated flavonoids but exhibits little lignification.

Although the *in vivo* function of POMT-2 remains to be determined, it is likely involved in the synthesis of lignin intermediates. *Populus* contains several flavonoids, including kaempferol and quercetin (Warren et al., 2003), but 6,7-dihydroxyflavone has not been reported in that genus. Here, POMT-2 showed only low activity against kaempferol and quercetin. Thus, its *in vivo* substrate may be caffeic acid. Alfalfa COMT shows preference to 3,4-dihydroxy, 5-methoxybenzaldehyde and protocatechuic aldehyde over caffeic acid (Kota et al., 2004). However, the down-regulation of COMT activity *in vivo* results in a change in lignin composition but does not affect the metabolism of benzaldehyde derivatives in that crop plant. Therefore, we suggest that an *in vitro* substrate preference is not always related to *in vivo* function of the gene product. This contradiction has also been found

with AtOMT1 from *Arabidopsis thaliana*. That particular flavonol 3′-OMT is based on an *in vitro* substrate assay (Muzac et al., 2000) with the recombinant protein, even though its *in vivo* function is that of lignin modification (Goujon et al., 2003). In conclusion, we have demonstrated here that POMT-2 encodes an O-methyltransferase that is likely involved with lignin biosynthesis in *P. deltoides*.

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