MOLECULAR CLONING AND CHARACTERIZATION OF TETRAHYDROPROTOBERBERINE cis-N-METHYLTRANSFERASE, AN ENZYME INVOLVED IN ALKALOID BIOSYNTHESIS IN OPIUM POPPY*  
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Running Title: Opium Poppy Tetrahydroprotoberberine cis-N-methyltransferase
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S-adenosyl-L-methionine: tetrahydroprotoberberine cis-N-methyltransferase (TNMT) [EC 2.1.1.122] catalyzes the conversion of (S)-stylopine to the quaternary ammonium alkaloid, (S)-cis-N-methylstylopine as a key step in the biosynthesis of protopine and benzophenanthridine alkaloids in plants. A full-length cDNA encoding a protein exhibiting 45% and 48% amino acid identity with coclaurine N-methyltransferase (CNMT) from Papaver somniferum (opium poppy) and Coptis japonica, respectively, was identified in an elicitor-treated opium poppy cell culture expressed sequence tag database. Phylogenetic analysis showed that the protein belongs to a unique clade of enzymes that includes CNMT, the predicted translation products of the Arabidopsis thaliana genes, At4g33110 and At4g33120, and bacterial SAM-dependent cyclopropane fatty acid synthases. Expression of the cDNA in Escherichia coli produced a recombinant enzyme able to convert the protoberberine alkaloids stylopine, canadine, and tetrahydropalmatine to their corresponding N-methylated derivatives. However, the protoberberine alkaloids tetrahydroxyberbine and scoulerine, and simple isoquinoline, benzylisoquinoline, and pavine alkaloids were not accepted as substrates demonstrating the strict specificity of the enzyme. The apparent \( K_m \) values for (R,S)-stylopine and S-adenosyl-L-methionine were 0.6 \( \mu \)M, and 11.5 \( \mu \)M, respectively. TNMT gene transcripts and enzyme activity were detected in opium poppy seedlings and all mature plant organs, and were induced in cultured opium poppy cells after treatment with a fungal elicitor. The enzyme was detected in cell cultures of other members of the Papaveraceae, but not in species of related plant families that do not accumulate protopine and benzophenanthridine alkaloids.

The benzylisoquinoline alkaloids are a large and diverse group of plant secondary metabolites, many of which possess potent pharmacological and other bioactive properties. Opium poppy (Papaver somniferum) is an extensively cultivated medicinal plant that produces more than 80 benzylisoquinoline alkaloids, including the narcotic analgesics morphine and codeine, and the antimicrobial sanguinarine. The effectiveness of sanguinarine as an inhibitor of fungal and bacterial growth suggests that it protects the plant against pathogens (1), and has prompted its use in oral hygiene products as an antiplaque agent (2). Sanguinarine biosynthesis begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde by norcoclaurine synthase (NCS) (3,4) to yield (S)-norcoclaurine (Fig. 1). Dopamine is formed by the decarboxylation of tyrosine and/or dihydroxyphenylalanine by tyrosine/dopa decarboxylase (TYDC) (5). Norcoclaurine 6-O-methyltransferase (6OMT) and coclaurine N-methyltransferase (CNMT) convert (S)-norcoclaurine to (S)-N-methylococlaurine (6-8). The P450-dependent monooxygenase (S)-N-methylococlaurine-3'-hydroxylase (CYP80B1) catalyzes the 3'-hydroxylation of (S)-N-methylococlaurine (9,10) prior to the formation of

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(S)-reticuline by 3'-hydroxy-N-methylcoclaunine 4'-O-methyltransferase (4'OMT) (8,11,12). (S)-Reticuline represents the last common intermediate in the biosynthesis of both sanguinarine and morphine (13). Berberine bridge enzyme (BBE) converts (S)-reticuline to (S)-scoulerine as the first committed step in the branch pathway leading to protopine and benzophenanthridine alkaloids, such as sanguinarine (Fig. 1). Alternatively, (S)-reticuline is epimerized to (R)-reticuline in the first steps of the morphinan alkaloid pathway in opium poppy. (S)-Scoulerine is converted to (S)-stylopine by the formation of two methylenedioxy bridges by the P450-dependent monoxygenases cheilanthifoline synthase and stylopine synthase (14,15). (S)-Tetrahydroprotoberberine cis-N-methyltransferase (TNMT) converts (S)-stylopine to (S)-cis-N-methylstylopine, which is subsequently hydroxylated by the P450-dependent monoxygenase, N-methylstylopine 14-hydroxylase (16). The initial reaction product tautomerizes to protopine, which is hydroxylated to yield dihydrosanguinarine by another P450-dependent monoxygenase, protopine 6-hydroxylase (17). Subsequent oxidation by dihydrobenzophenanthridine oxidase yields sanguinarine (18).

Several S-adenosyl-L-methionine (SAM)-dependent O-methyltransferases involved in the biosynthesis of a variety of plant secondary metabolites have been characterized at the molecular level (7,11,12,19,20) and structural levels (21-23). However, few studies have focused on the molecular biochemical characterization of SAM-dependent N-methyltransferases (24-27). At least two N-methyltransferases are involved in benzylisoquinoline alkaloid biosynthesis (Fig. 1). CNMT has been purified (28), and corresponding cDNAs isolated from Coptis japonica (6), Thalictrum flavum (29), and Papaver somniferum (8). TNMT protein has been isolated from Eschscholzia californica and Corydalis vaginans cell cultures (30), and purified from Sanguinaria canadensis cell cultures (31). However, a cDNA encoding TNMT has not been reported. TNMT catalyzes the SAM-dependent methylation of the tertiary nitrogen of (S)-stylopine to produce the quaternary ammonium compound (S)-cis-N-methylstylopine, which is the ultimate precursor to protopine and the benzophenanthridine alkaloids (32). The transfer of a methyl group to a tertiary nitrogen atom is a relatively rare reaction in plant metabolism. Only two cDNAs encoding plant N-methyltransferases that catalyze the formation of low molecular weight quaternary ammonium compounds have been reported (33,34). Both of these enzymes are involved in the biosynthesis of the osmoprotective metabolite betaine.

We report the identification and functional characterization of a cDNA encoding TNMT from opium poppy. TNMT is the only SAM-dependent N-methyltransferase in plant alkaloid metabolism known to produce a quaternary ammonium compound, and the first enzyme specific to protopine and benzophenanthridine alkaloid biosynthesis to be characterized at the molecular biochemical level.

**EXPERIMENTAL PROCEDURES**

**Plant material** — Opium poppy (Papaver somniferum cv. Marianne) plants were cultivated in growth chambers as described previously (8). Cell suspension cultures were maintained at 23°C under continuous fluorescent light with a flux rate of 35 μmol s⁻¹ m⁻² on 1B5 medium (35) consisting of B5 salts and vitamins, 1g L⁻¹ hydroyzed casein, 20 g L⁻¹ sucrose, and 1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (8). *Papaver somniferum* and *P. bracteatum* cell cultures were treated with an elicitor prepared from Botrytis cinerea mycelia as described previously (8).

**Chemicals** — (R,S)-Tetrahydropalmatine (80% w w⁻¹) from Corydalis yahusuo was purchased from Ethnogarden Botanical (Barrie, Ontario, Canada). (R,S)-Tetrahydroberberine [(R,S)-canadine], DL-pavine hydrochloride, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, and 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). (S)-Scoulerine was purchased from Indofine Chemical (Hillsborough, NJ, USA). S-Adenosyl-L-[methyl-¹⁴C]-methionine (1850 GBq mol⁻¹) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich.

**Chemical synthesis of 2,3,9,10-tetrahydroxyberbine** — (R,S)-
Tetrahydropalmatine was demethylated using 6 molar equivalents of BBr₃ according to the procedure of McOmie et al. (36). The product was identified by low-resolution EI-MS (m/z: 299 [26], 178 [47], 164 [40], 162 [47], 136 [13]), and the loss of O-methyl groups was confirmed by ¹H-NMR.

Chemical synthesis of stylopine — (R,S)-Stylopine was synthesized from 2,3,9,10-tetrahydroxyberbine based on the method of Castillo et al. (37). Briefly, a solution of 1 g 2,3,9,10-tetrahydroxyberbine (HMPT) was added to a stirred solution of 0.4 g NaH in 16 ml HMPT over 10 min. Subsequently, CH₂Cl₂ (0.9 g) was added and the solution stirred for 20 min. The reaction was quenched with ice water (50 ml) and extracted with Et₂O (3 x 15 ml). The extract was dried over MgSO₄ and concentrated under reduced pressure. Stylopine was precipitated with the addition of CHCl₃/MeOH, and collected by vacuum filtration through a 0.5 µm FH membrane (Millipore, Bedford, Massachusetts). Product identity was confirmed by low-resolution EI-MS (m/z: 323 [30], 179 [15], 174 [11], 148 [100]), and was in agreement with previous reports (38-40).

Nucleic acid isolation and analysis — The synthesis and sequencing of an elicitor-treated opium poppy cell culture cDNA library, and the assembly of the corresponding expressed sequence tag (EST) database were described previously (41). A full-length cDNA encoding TNMT was identified by screening the EST database in silico using the tBLASTn algorithm (42) in DNATools 6.0 (http://www.crc.dk/dnataools). CNMT cDNAs from P. somniferum (8), Thalictrum flavum (29), and Coptis japonica (6) were used as query sequences. Total RNA was isolated using the method of Cheng and Seemann (43). Gel-blot hybridization analysis was performed as described previously (3), using a random-primer ³²P-labeled probe derived from the full-length TNMT cDNA.

Heterologous expression of TNMT and At4g33120 cDNAs — The TNMT open reading frame (ORF) was amplified using the full-length cDNA as a template to amplify the corresponding ORF. The sense primer contained a BamHI site and was specific for the 5′-end of the ORF (4E9-BamHI; 5′-TAGGATCCATGGGTTCAATAGATG-3′), whereas the antisense primer contained an XhoI site and was complimentary to the 3′-end (4E9Term; 5′-TACAGCTAGCTCTTTTGTGAAAAGCAGC-3′). A full-length cDNA of the Arabidopsis thaliana gene At4g33120 (stock number U22568) was obtained from the Arabidopsis Biological Resource Center (Columbus OH, USA) and used as a template to amplify the corresponding ORF. The sense primer contained a BamHI site and was specific for the 5′-end of the ORF (AtTNMT-F; 5′-ATGCGATCCATGGGAAAGATTATGGC-3′), whereas the antisense primer contained an EcoRI site and was complimentary to the 3′-end (AtTNMT-R; 5′-TACAGAATTTCATTCTTTCTTCTTTGAAGAGCAGC-3′). Amplicons were ligated into pRSETB (Invitrogen) using the engineered restriction sites to produce pTNMT and pDLAt120, respectively. Escherichia coli strain ER2566 pLysS (New England Biolabs, Beverly, MA, USA) was transformed with pTNMT or pDLAt120. Cultures were shaken at 30°C in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) to an A₆₀₀ of 0.6 and subsequently induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4.5 h at 30°C. Cells were harvested by centrifugation at 5000g for 5 min. E. coli strain Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) was transformed with pTNMT or pDLAt120. Cultures were shaken at 30°C in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) to an A₆₀₀ of 0.6. Cultures were induced with 0.2 mM IPTG for 17 h at 22°C and harvested at 5000g for 5 min. Bacterial pellets were resuspended in buffer A (100 mM Tris-HCl, pH 7.8, 12 mM β-mercaptoethanol) containing pepstatin A (5 µM) and leupeptin (5 µM), and lysed using a French press at 15,000 psi. Lysates were cleared by centrifugation at 10,000g for 20 min and desalted on PD-10 columns (Amersham Biosciences, Piscataway, NJ, USA).

Purification of recombinant TNMT — E. coli ER2566 pLysS cells harboring the pTNMT plasmid were used to produce recombinant TNMT, which was precipitated from the crude bacterial lysate using 20-40% (w/v) ammonium sulfate and resuspended in binding buffer (20 mM NaPO₄, pH 7.4; 500 mM NaCl). After
desalting, recombinant (poly)histidine-tagged TNMT was purified by nickel affinity chromatography using a HiTrap Chelating HP column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer’s instructions, and concentrated by ultrafiltration.

**Immunoblot Analysis** — Soluble proteins (25 μg) were fractionated by SDS-PAGE and transferred to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA). Protein blots were incubated with mouse monoclonal anti-(poly)histidine clone HIS-1 (Sigma, St. Louis, MO, USA) diluted 1:3000 in Blotto (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% [w v⁻¹] milk protein) for 12 h, washed in TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% [v v⁻¹] Tween 20), and incubated for 1 h with alkaline phosphatase (AP)—conjugated anti-mouse secondary antibodies (Bio-Rad, Hercules, CA, USA). The membranes were washed in TBST and developed in AP buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM MgCl₂) containing 20 μM nitroblue tetrazolium and 20 μM 5-bromo-4-chloro-3-indolyl phosphate as substrates (44).

**Enzyme assays and characterization using plant or recombinant proteins** — Plant tissues were ground to a fine powder under liquid nitrogen using a mortar and pestle. Powdered tissues were extracted in buffer A containing 100 mM NaCl; 5 mM MgCl₂ containing 20 μM nitroblue tetrazolium and 20 μM 5-bromo-4-chloro-3-indolyl phosphate as substrates (44). The aqueous phase was evaporated to dryness and the organic fraction was subsequently concentrated under reduced pressure and extracted twice with 1 M HCl. The aqueous phase was evaporated to dryness and analyzed by low-resolution El-MS. The resulting mass spectrum (m/z: 369 [3], 355 [71], 192 [9], 190 [26], 164 [100], 149 [69]) was in agreement with that of N-methyltetrahydropalmatine (39,46,47), indicating that N-methyl transfer had occurred.

**Phylogenetic analysis** — Amino acid alignments were performed using the CLUSTALW (48) algorithm in MegAlign (DNAStar, Inc.). The neighbour-joining phylogeny was generated and bootstrap analysis performed with TREECON (49). GenBank accession numbers for the sequences used are: *Thalictrum flavum* CNMT (AAU20766); *Mycobacterium tuberculosis* PcaA (ZP_00880145); *Coptis japonica* CNMT (BAB71802); *Papaver somniferum* CNMT (AAP45316); *Mesorhizobium loti* CFAPS (BAB53730); *Burkholderia vietnamiensis* CPFAS (ZP_00420926); *Arabidopsis thaliana* At4g333110 ORF (AAM65762); *Arabidopsis thaliana* At4g333120 ORF (NP_195038); *Thalictrum flavum* SOMT (AAU20770); *Coptis japonica* SOMT (BA06192); *Lycopersicon esculentum* PEANMT (AAG59894); *Coffea arabica* DMXNMT1 (BAC75663); *Coffea
Several plant-derived and SAM-dependent O- and N-methyltransferases were subjected to phylogenetic analysis to examine the possible evolutionary relationships among these enzymes (Fig. 2). High bootstrap support was indicated for a monophyletic clade that included PsTNMT, coarlaunine N-methyltransferases (PsCNMT, CjCNMT, TFCNMT), bacterial SAM-dependent cyclopropane fatty acid synthases (MtpA, AafA, BhCPFAS), and the predicted translation products of the Arabidopsis thaliana genes At4g33110 and At4g33120. Strong support was also observed for a separate monophyletic clade including purine N-methyltransferases from Coffea arabica and Camellia sinensis (CaDMXNMT, CaMXNMT, CsDMXNMT), and salicylic acid carboxyl methyltransferase from Clarkia breweri (CbSAMT). Putrescine N-methyltransferases from Datura stramonium and Atropa belladonna (DsPNMT, AbPNMT) formed a distinct monophyletic clade with strong support. Notably, opium poppy tetrahydroprotoberberine cis-N-methyltransferase (PsTNMT), Lycopersicon esculentum phosphoethanolamine N-methyltransferase (LePANMT), and Limonium latifolium β-alanine N-methyltransferase (LIBANMT) all catalyze the SAM-dependent methylation of a tertiary amine in the formation of a quaternary ammonium compound, yet these enzymes are unrelated in this phylogeny indicating that they evolved independently.

The amino acid sequence alignment shown in Fig. 3 compares PsTNMT to other members of its phylogenetic clade (Fig. 2). As expected, opium poppy TNMT shares considerable sequence identity with CNMT proteins from P. somniferum (48%), T. flavum (46%), and C. japonica (45%). The predicted translation products of At4g33110 and At4g33120 (Fig. 3) also exhibit considerable (44%) sequence identity to opium poppy TNMT. Surprisingly, a representative SAM-dependent mycolic acid cyclopropane synthase from Mycobacterium tuberculosis, MtPeaA, shows 21-24% identity to plant TNMT and CNMTs. The crystal structure of MtPeaA in complex with S-adenosylhomocysteine (SAH), the by-product of SAM-dependent methylation, has revealed...
the amino acid residues responsible for interaction with the SAM cofactor (51). Several of these critical SAM-binding residues, marked by asterisks in Fig. 3, are conserved in plant N-methyltransferases involved in benzylisoquinoline alkaloid biosynthesis.

**Heterologous Expression of TNMT** — The identity of the isolated cDNA as TNMT was confirmed by the production of recombinant protein in *E. coli* ER2566 pLysS harboring the TNMT expression construct pTNMT. The recombinant enzyme displayed an apparent molecular weight of approximately 48 kDa, which was expected due to N- and C-terminal peptide fusions derived from the expression vector (Fig. 4A,B). The recombinant (poly)histidine-tagged TNMT was detected only in total protein extracts from *E. coli* harboring the pTNMT plasmid (Fig. 4B). Using (R,S)-stylopine as the alkaloid substrate, TNMT activity was detected only in soluble protein extracts of *E. coli* expressing the full-length TNMT cDNA (Fig. 4C).

Due to substrate availability, the product of the enzyme-catalyzed reaction was investigated using (R,S)-tetrahydropalmatine. A large-scale enzyme assay containing approximately 1 mg of total soluble protein from *E. coli* harboring pTNMT, 2.5 µmol of (R,S)-tetrahydropalmatine, and 2.5 µmol of unlabeled SAM was incubated overnight at 37°C. The reaction mixture was extracted with ethyl acetate, acidified, and the resulting aqueous phase evaporated to dryness. EI-MS analysis (Supplemental data; Fig. S2) revealed the expected mass spectrum for N-methyltetrahydropalmatine (39,46,47) confirming that the isolated cDNA encodes TNMT.

Although TNMT polypeptide accumulation was most abundant in IPTG-induced cultures of *E. coli* harboring pTNMT (Fig. 4A,B), the recombinant enzyme was also detected in total protein extracts of control cultures harboring pTNMT (Fig. 4A,B). However, markedly higher levels of TNMT activity were detected in soluble protein fractions from *E. coli* cultures not treated with IPTG (Fig. 4C). Neither (poly)histidine-tagged protein accumulation nor TNMT activity were detected in extracts of cells containing the empty pRSETB vector (Fig. 4B,C).

(Poly)histidine-tagged, recombinant TNMT was purified by nickel-affinity chromatography, as shown in Fig. 4A. Although the purified protein was active, TNMT activity was difficult to stabilize after nickel-affinity chromatography due to the sensitivity of the recombinant opium poppy enzyme to divalent cations. The addition of 1 mM chloride salts of Ni²⁺, Co²⁺, Ca²⁺ or Mn²⁺ to enzyme assays using 2 µg of 20-40% (w v⁻¹) ammonium sulfate-precipitated protein from *E. coli* cultures harboring pTNMT inhibited N-methyltransferase activity by 72%, 71%, 62%, and 41%, respectively. This inhibition could be mostly prevented by the inclusion of 10 mM EDTA in the assay mixture. In contrast, the addition of 1 mM MgCl₂ to the reaction mixture had no effect on TNMT activity. Our data are in agreement with a previous report that TNMT from *Sanguinaria canadensis* was also inhibited by Co²⁺, Ca²⁺, and Mn²⁺ ions (31).

The *A. thaliana* protein encoded by the At4g33120 gene was also produced in *E. coli* and tested for TNMT activity. No activity was detected when (R,S)-stylopine, (R,S)-tetrahydropalmatine or dimethoxytetrahydroisooquinoline were used as potential substrates (data not shown).

**Enzymatic properties of recombinant TNMT** — The pH optimum for TNMT activity was 7.5, and half-maximal activity was estimated to occur at 6.8 and 8.2 (Fig. 5A). Recombinant TNMT showed maximum activity at 37°C and half-maximal activity would be expected at 12°C and 47°C (Fig 6B). Varying the concentration of (R,S)-stylopine from 0.5 to 32 µM at a saturating concentration (20 µM) of SAM, or the concentration of SAM from 0.625 to 40 µM, at a saturating concentration (50 µM) of (R,S)-stylopine, produced typical Michaelis-Menten substrate saturation kinetics. Apparent *Kₘ* values for (R,S)-stylopine and SAM of 0.6 µM and 11.5 µM, respectively were calculated from Lineweaver-Burk plots (Fig. 5C,D). The incorporation of radioactivity from [methyl-¹⁴C]-SAM was used to demonstrate the strict substrate specificity of recombinant TNMT (Fig. 6). Only the protoberberine alkaloids (R,S)-stypoline, (R,S)-canadine, and (R,S)-tetrahydropalmatine were accepted as substrates. No radiolabelled products were detected when the simple isoquinoline alkaloids dimethoxytetrahydroisooquinoline and
methylisooquinolinediol, the benzylisoquinoline alkaloid norlaudanosoline, the protoberberine alkaloids (R,S)-tetrahydroxyberbine and (S)-scoulerine, or the pavine alkaloid (R,S)-pavine were tested as substrates (Fig. 6).

Developmental and inducible gene expression—RNA gel-blot hybridization analysis showed that TNMT gene transcripts accumulate most abundantly in roots, to a lesser extent in leaves, and at lower levels in stems and flower buds (Fig. 7A). In contrast, the highest levels of TNMT activity were measured in stem and leaf tissues, with lower levels detected in roots and flower buds (Fig. 7B). Neither TNMT transcripts (Fig. 7C) nor enzyme activity (Fig. 7D) were detected in opium poppy seedlings one day after seed imbibition. However, both TNMT transcript levels and enzyme activity were detected 4 d after imbibition, and remained relatively constant during seedling growth. TNMT transcripts were not detected in control opium poppy cell cultures, but were rapidly induced within 2 h after elicitor treatment (Fig. 7E). Subsequently, TNMT transcript levels gradually decreased between 10 and 100 h after elicitor treatment (Fig. 7E). In contrast, TNMT activity increased from basal levels to a maximum 50 h post-elicitation, followed by a gradual decrease between 50 and 100 h (Fig. 7F).

Occurrence of TNMT activity in other benzylisoquinoline alkaloid-producing species — Cell cultures of several benzylisoquinoline-producing plants representing four different plant families were assayed for TNMT activity by measuring the incorporation of radioactivity from [methyl-14C]-SAM when (R,S)-stylopine was supplied as a substrate (Fig. 9). (R,S)-stylopine can only be methylated at the lone tertiary nitrogen. Alkaloid production was measured in cell cultures of Papaver somniferum and P. bracteatum, which do not normally produce alkaloids under these conditions, by treatment with elicitor prepared from mycelia of Botrytis cinerea (8). Cell cultures of the other plant species tested produced alkaloids constitutively. Protoberberine and/or benzophenanthridine alkaloid accumulation was confirmed in these cell cultures by TLC analysis using authentic alkaloids as standards (data not shown). TNMT activity was detected in elicitor-treated cell cultures of Papaver somniferum and P. bracteatum, and untreated cultures of Eschscholzia californica, Sanguinaria canadensis, Chelidonium majus, and Argemone mexicana (Fig. 8), which are all members of the Papaveraceae. Little or no TNMT activity was detected in cell cultures of Nandina domestica, Tinospora cordifolia, Thalictrum flavum, which represent the Berberidaceae, Menispermaceae, and Ranunculaceae, respectively (Fig. 8). TNMT activity was also not detected in Nicotiana tabacum, which was used as a negative control.

DISCUSSION

We report the molecular cloning and characterization of a full-length cDNA encoding TNMT from opium poppy. This novel SAM-dependent N-methyltransferase was identified by tBLASTn analysis of an elicitor-treated opium poppy cell culture EST database using CNMTs as query sequences. Functional expression of the TNMT cDNA in E. coli showed that the (poly)histidine-tagged enzyme catalyzed the conversion of certain tetrahydroprotoberberine alkaloids to their corresponding N-methylated quaternary ammonium derivatives. Catalytic function was confirmed through purification to homogeneity of the (poly)histidine-tagged protein (Fig. 4). Substantially more soluble and active recombinant enzyme was produced in E. coli strain Rosetta (DE3) pLysS compared with E. coli strain ER2566 pLysS due to the occurrence of 29 rare bacterial codons in the TNMT cDNA. Moreover, bacterial cultures grown in the absence of IPTG showed a reduced rate of TNMT synthesis, which increased the proper assembly of the active enzyme (Fig. 4).

The catalytic properties of recombinant opium poppy TNMT are generally in agreement with those reported for the purified or partially purified enzyme from related plants. The predicted molecular weight of opium poppy TNMT (40.8 kDa) is comparable to that of the purified, denatured enzyme from Sanguinaria canadensis (39 kDa) determined by SDS-PAGE (31). However, gel-filtration chromatography of TNMT in purified partially purified protein extracts from S. canadensis, Eschscholzia californica, and Corydalis vaginans revealed native molecular weights of 70 kDa, 78 kDa, and 72 kDa, respectively (30,31), suggesting that native TNMT is a homodimer. The temperature and pH optima for recombinant opium poppy
TNMT (Fig. 5) are similar to those reported for native TNMT from *S. canadensis*, *E. californica*, and *C. vaginans* (30,31). TNMT has been reported to specifically utilize (S)-enantiomers of tetrahydroprotoberberine substrates. Although we were unable to address the enantiomeric selectivity of opium poppy TNMT, the $K_m$ value determined for (R,S)-styloipone (0.6 $\mu$M) would reflect a $K_m$ for (S)-styloipone of 0.3 $\mu$M if (R)-styloipone was not an accepted substrate. This is lower than the $K_m$ values for (S)-styloipone of 3.1 and 4.0 $\mu$M reported for TNMT from *E. californica* and *C. vaginans* (30), respectively. Opium poppy TNMT exhibited an apparent $K_m$ for SAM of 11.5 $\mu$M, which is comparable to that reported for TNMT from *E. californica* (30). In contrast, opium poppy TNMT displayed a $K_m$ value for SAM approximately 10-fold higher than those determined for TNMT from *S. canadensis* and *C. vaginans* (30,31). Opium poppy TNMT displayed a narrow substrate range with only tetrahydroprotoberberine substrates possessing dimethoxy (i.e., tetrahydropalmatine and canadine) or methylenedioxy (i.e., styloipone and canadine) functionalities at C-2/C-3 and C-9/C-10 as acceptable substrates (Fig. 6). The same substrate specificity profile was reported for native TNMT from *E. californica* and *C. vaginans* (30).

Phylogenetic analysis supports a monophyletic origin for the two known N-methyltransferases involved in benzylisoquinoline alkaloid biosynthesis (Fig. 2). The downstream metabolic role suggests that TNMT resulted from gene duplication after the more ancient recruitment of CNMT. This is supported by the widespread occurrence of TNMT activity in the Papaveraceae, but not in members of plant families that accumulate benzylisoquinoline, but not specifically protopine or benzophrantheridine alkaloids (Fig. 8). The predicted translation product of the *A. thaliana* gene At4g33120, a close homolog of CNMT (6) and TNMT, did not catalyze the N-methylation the simple isoquinoline dimethoxytetrahydroisoquinoline, which was accepted by *C. japonica* CNMT (28), or the protoberberine alkaloids (R,S)-styloipone and (R,S)-tetrahydropalmatine, which are substrates of TNMT (Fig. 6). The similarity of the *A. thaliana* At4g33110 and At4g33120 gene products to CNMT and TNMT potentially reveals a latent genetic fingerprint for alkaloid biosynthesis in a plant species no longer able to produce these secondary metabolites (4). TNMT is a unique plant enzyme that catalyzes the formation of quaternary ammonium compounds. Aside from specialized protein lysine N-methyltransferases (52), only two cDNAs encoding plant N-methyltransferases that catalyze the formation of low molecular weight quaternary ammonium compounds have been reported. However, both phophethanolamine N-methyltransferase (24) and \(\beta\)-alanine N-methyltransferase (26) are involved in the biosynthesis of betaines, which are important osmoprotectants in high salinity environments (33,34). These enzymes, along with other N-methyltransferases involved in the biosynthesis of caffeine and other alkaloids (24,26,27), do not share a close phylogenetic relationship with TNMT (Fig. 2).

Although TNMT and CNMTs share limited sequence similarity with cyclopropane synthases, several residues known to be involved in SAM binding in MtPcaA are strictly conserved (Fig. 3). The crystal structure of MtPcaA in complex with SAH demonstrates how Ser-34 shares a hydrogen bond with a carboxylate oxygen, and Gly-72 and Ile-136 interact with the nitrogen of the amino acid portion of SAM. Moreover, Gly-74, Thr-94, and Gln-99 are known to share hydrogen bonds with ribosyl hydroxyl moieties (51). The glycine-rich sequence containing Gly-72 and Gly-74 (E/DXGXXG), often referred to as motif I (Fig. 3), is highly conserved in many SAM-dependent methyltransferases, and is also present in TNMT, CNMTs and MtPcaA (6,51,53). Structure and sequence conservation shows that MtPcaA exhibits a structural fold most similar to the small molecule sub-class of methyltransferases (53). The conservation of critical residues involved in SAM binding suggests that TNMT and CNMTs also maintain this core small molecule methyltransferase fold. A methyltransferase fold model also predicts that SAM-binding motifs reside in the N-terminal domains of TNMT and CNMTs. As such, amino acid residues that confer substrate specificity are likely located in the C-terminal regions of these proteins (53). In the absence of crystal structures for CNMTs and TNMT, residues that confer substrate and reaction
specificity with respect to secondary or tertiary nitrogen methylation might be identified among C-terminal residues that are conserved in CNMTs, but unique to TNMT.

The incongruity between TNMT gene transcript abundance and enzyme activity in opium poppy organs suggests this step in alkaloid metabolism might be subject to translational and/or post-translational regulation. The detection of TNMT gene transcripts and enzyme activity in roots (Fig. 5A,B) supports the biosynthesis of the protopine and benzophenanthridine alkaloids, which accumulate predominantly in this organ (54). However, the relatively high level of TNMT gene transcripts and enzyme activity in other plant organs suggests an involvement in additional alkaloid biosynthetic pathways. Noscapine and narcotine are major benzylisoquinoline alkaloids found in all opium poppy organs (54) and similar phthalideisoquinolines are found in other members of the Papaveraceae and Menispermaceae (4). Noscapine has been used as an antitussive agent and recently purported to possess anti-cancer properties (55). The biosynthesis of phthalideisoquinoline alkaloids remains poorly understood, although (S)-scoulerine and (S)-canadine been proposed as noscapine pathway intermediates (56,57). The conversion of (S)-scoulerine to (S)-canadine involves an O-methyltransferase (58) and a methylenedioxy bridge-forming cytochrome P450 (59). The preference of (R,S)-canadine as a substrate (Fig. 6) and the occurrence of the enzyme in organs that accumulate abundant noscapine, but not sanguinarine, suggests that TNMT also participates in the biosynthesis of noscapine since both (S)-N-methylcanadine and noscapine possess N-methyl, 2,3-methylenedioxy, and 9,10-dimethoxy substitutions (57). BBE gene transcripts and enzyme activity, required for (S)-scoulerine formation, have also been reported in aerial organs of opium poppy (60).

In opium poppy seedlings, TNMT transcripts and enzyme activity were first detected 4 d after seed imbibition, which correlates the accumulation of sanguinarine approximately 5 d after germination (9). The rapid activation of TNMT supports a key defensive role for various benzylisoquinoline alkaloids early in plant development. The inducible accumulation of TNMT transcripts in elicitor-treated cell cultures (Fig. 7) reflects the coordinated temporal induction of all other known genes involved in sanguinarine biosynthesis in opium poppy (8). Moreover, the accumulation profiles of N-methylstyloline (41), other benzophenanthridine alkaloid pathway intermediates (41), and sanguinarine (41,60) are in agreement with the temporal induction of TNMT activity in elicitor-treated opium poppy cell cultures.

The molecular cloning and characterization of opium poppy TNMT adds an important catalytic functionality to the repertoire of enzymes involved in benzylisoquinoline alkaloid biosynthesis. The phylogenetic relationship between CNMT and TNMT provides new insights into the evolutionary recruitment of enzymes into plant alkaloid pathways. The availability of the TNMT cDNA also provides an opportunity to elucidate the catalytic mechanism of tertiary vs. secondary nitrogen methylation via the comparative structural biology of two unique, yet related SAM-dependent N-methyltransferases.

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**FOOTNOTES**

* The nucleotide sequence for the opium poppy TNMT cDNA has been deposited in the GenBank database under Accession Number DQ028579. We thank Dr. Natalia Loukanina and Dr. Jörg Ziegler for helpful discussions. D.K.L. is the recipient of a Natural Sciences and Engineering Research Council of Canada (NSERC) postgraduate scholarship. P.J.F. holds the Canada Research Chair in Plant Metabolic Processes Biotechnology. This work was funded by NSERC Strategic and Discovery grants to P.J.F.

**FIGURE LEGENDS**

**Fig 1.** Protopine and benzophenanthridine alkaloid biosynthesis in opium poppy. Biosynthetic enzymes for which corresponding genes have been isolated are labeled. Abbreviations: 4’OMT, (S)-3’-hydroxy-N-methylecoclaurine 4’-O-methyltransferase; 6OMT, (S)-norcoclaurine 6-O-methyltransferase; BBE, berberine bridge enzyme; CNMT, (S)-coclaurine N-methyltransferase; CYP80B1, (S)-N-methylecoclaurine 3’-hydroxylase; NCS, (S)-norcoclaurine synthase; TNMT, (S)-tetrahydroprotoberberine cis-N-methyltransferase; TYDC, tyrosine decarboxylase.

**Fig 2.** Neighbour-joining tree derived from selected plant O- and N-methyltransferases. Amino acid sequences were aligned using ClustalW (48). The tree was constructed and bootstrap analysis performed using TREECON (49). Internal labels give bootstrap frequencies for each clade as percentages of 1000 iterations. GenBank accession numbers are listed in Experimental Procedures.

**Fig 3.** Alignment of the deduced amino acid sequence of *Papaver somniferum* TNMT with *P. somniferum* CNMT, *Coptis japonica* CNMT, *Thalictrum flavum* CNMT, the *Arabidopsis thaliana*
At4g33120 gene product, and *Mycobacterium tuberculosis* PcaA, a SAM-dependent cyclopropane synthase. Residues identical to TNMT have black backgrounds; residues that are similar to TNMT are shaded in grey. Asterisks (*) denote conserved residues known to be involved in S-adenosyl-L-methionine binding (51). The conserved SAM-dependent methyltransferase region, motif I (53), is underlined. Sequence alignment was performed using ClustalW (48). GenBank accession numbers are listed in Experimental Procedures.

Fig 4. Heterologous expression of the TNMT cDNA in *Escherichia coli*. A, SDS-PAGE analysis of total protein extracts (25 μg) from non-induced (-IPTG) and induced (+IPTG) *E. coli* cells harboring the pRSETB empty vector control, or the pTNMT expression vector. Purified, recombinant TNMT is also shown. B, Western blot analysis performed on protein samples from A using a (poly)histidine tag monoclonal antibody shows the occurrence of recombinant proteins. Numbers on the left in A and B show the migration and molecular weight of standard protein markers. C, TNMT activity was assayed in total soluble protein extracts (10 μg).

Fig 5. General enzymatic properties of recombinant TNMT. A, Effect of pH on TNMT activity. B, Effect of temperature on TNMT activity. C, Double-reciprocal plots showing the effect of substrate concentration on the reaction velocity for (R,S)-stylopine. D, Double-reciprocal plots showing the effect of substrate concentration on the reaction velocity for S-adenosyl-L-methionine. The apparent $K_m$ values for (R,S)-stylopine and S-adenosyl-L-methionine were calculated as the negative reciprocals of the x-intercepts in C and D, respectively.

Fig 6. Substrate specificity of recombinant TNMT. The assay mixtures contained 100 mM Tris-HCl (pH 7.8), 12 mM β-mercaptoethanol, 1 mM EDTA, 2 μM [methyl-14C]-S-adenosyl-L-methionine, 500 μM alkaloid substrate, and 5 μg of protein. Assays were incubated for 30 min at 37°C. Abbreviation: nd, not detected.

Fig 7. Relative abundance of *TNMT* gene transcripts (A, C, and E) and TNMT enzyme activity (B, D, and F) in organs (A and B), developing seedlings (C and D), and elicitor-treated cell cultures (E and F) of opium poppy. RNA gel-blot hybridization analysis was performed using total RNA (15 μg), which was fractionated, transferred to a nylon membrane, and hybridized at high stringency to the 32P-labelled TNMT cDNA. Gels were stained with ethidium bromide prior to blotting to ensure equal loading. Error bars represent the standard error of the mean for three independent measurements.

Fig 8. TNMT activity in cell cultures of various benzylisoquinoline alkaloid-producing plant species. Assays contained 2 μg of total soluble protein extract, 500 μM (R,S)-stylopine, and 2 μM [methyl-14C]-S-adenosyl-L-methionine, and were incubated for 20 min at 37°C.
Figure 2
### Figure 6

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David K. Liscombe and Peter J. Facchini

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