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Molecular Cloning of an *O*-Methyltransferase from Adventitious Roots of *Carapichea ipecacuanha*

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Carapichea ipecacuanha **produces various emetine-type alkaloids, known as ipecac alkaloids, which have long been used as expectorants, emetics, and amebicides. In this study, we isolated an *O*-methyltransferase cDNA from this medicinal plant. The encoded protein (CiOMT1) showed 98% sequence identity to IpeOMT2, which catalyzes the 7′-*O*-methylation of 7′-*O*-demethylcephaeline to form cephaeline at the penultimate step of emetine biosynthesis (Nomura and Kutchan, *J. Biol. Chem.*, 285, 7722–7738 (2010)). Recombinant CiOMT1 showed both 7′-*O*-methylation and 6′-*O*-methylation activities at the last two steps of emetine biosynthesis. This indicates that small differences in amino acid residues are responsible for distinct regional methylation specificities between IpeOMT2 and CiOMT1, and that CiOMT1 might contribute to two sequential *O*-methylation steps from 7′-*O*-demethylcephaeline to emetine.**

Key words: *O*-methyltransferase; ipecac alkaloid biosynthesis; cephaeline; 7′-*O*-demethylcephaeline

Carapichea (synonyms in Genbank taxonomy: *Cephaelis, Psychotria*) *ipecacuanha* (Brot.) A. Rich. (Rubiaceae), commonly known as Rio or Brazilian ipecac, is a perennial shrub 20–40 cm in height that is native to Brazil, particularly the moist and shady forests of Matto Grosso and Minas Geraes. It is also cultivated to some extent in Malaysia, Burma, and the Darjeeling Hills of West Bengal, India.[1)] Ipecac is used as an expectorant in the treatment of bronchitis, croup, asthma, and whooping cough, as an emetic in cases of poisoning, and as an amebicide treating in amoebic dysentery. It is rich in the terpenoid isoquinoline alkaloids (also called ipecac alkaloids) emetine, cephaeline, psychotrine, *O*-methylpsychotrine, and emetamine, and emetine and cephaeline are the principal active pharmaceuticals.

Since ipecac plants are endangered due to human disturbance of their natural environment, *in vitro* cultures of it have been intensively investigated to produce cephaeline and emetine, but the desired alkaloids are still obtained in yields that are inadequate for industrial production.[2)] Whereas metabolic engineering in alkaloid biosynthesis has also been explored to improve both the quantity and the quality of several alkaloids,[3)] biosynthetic enzymes and their genes in ipecac alkaloids are still limited, except for the recent isolation of glycosidases and *O*-methyltransferases.[4,5)]

Ipecac alkaloids are synthesized from the condensation of dopamine derived from tyrosine, for isoquinoline moieties, and from secologanin, as a monoterpenoid molecule (Fig. 1). This Pictet-Spengler-type condensation yields two epimers, (*R*)-*N*-deacetylipecoside and (*S*)-*N*-deacetylisoipecoside. The (*R*)-epimer is further converted to alangiside-type glucosides *via* deacetylipecoside synthase, whereas the (*S*)-epimer is converted to various ipecac alkaloids *via* deacetylisoipecoside synthase.[6)] *N*-Deacetylisoipecoside is further converted by *O*-methyltransferases (OMT) and glycosidases to produce protoemetine. Protoemetine is condensed with dopamine and then converted to cephaeline and emetine by OMTs.

As shown in Fig. 1, *O*-methyltransferases (OMTs) play important roles in the biosynthesis of secondary metabolites, including alkaloids, since the *O*-methylation of polyhydroxylated small molecules is crucial in directing intermediates to a specific biosynthetic pathway.[7,8)] *O*-Methylation occurs actively at the 6- and 7-hydroxyl groups of the isoquinoline moieties (Fig. 1) of ipecac alkaloids, as seen in benzylisoquinoline alkaloid biosynthesis in *Coptis japonica*.[9)] Based on the possibility that ipecac OMTs in ipecac alkaloid biosynthesis resemble those in benzylisoquinoline alkaloid biosynthesis, we started to isolate candidate OMT genes in ipecac alkaloid biosynthesis using a homology-based cloning approach, and characterized the enzymological properties of recombinant CiOMT1. Whereas Nomura and Kutchan[5)] recently reported the isolation of ipecac OMTs (IpeOMTs) in ipecac alkaloid biosynthesis, our isolated enzyme showed slightly different reaction specificities with several amino acid substitutions, as compared to IpeOMTs. Below, we discuss the role of CiOMT1 in ipecac alkaloid biosynthesis and the molecular evolution of ipecac OMTs.

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; Kpi buffer, potassium phosphate buffer; LC-MS, liquid chromatography-mass spectroscopy; NAA, naphthalene acetic acid; OMT, *O*-methyltransferase; 6-OMT, norcoclaurine 6-*O*-methyltransferase; 4′-OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase; TFA, trifluoroacetic acid

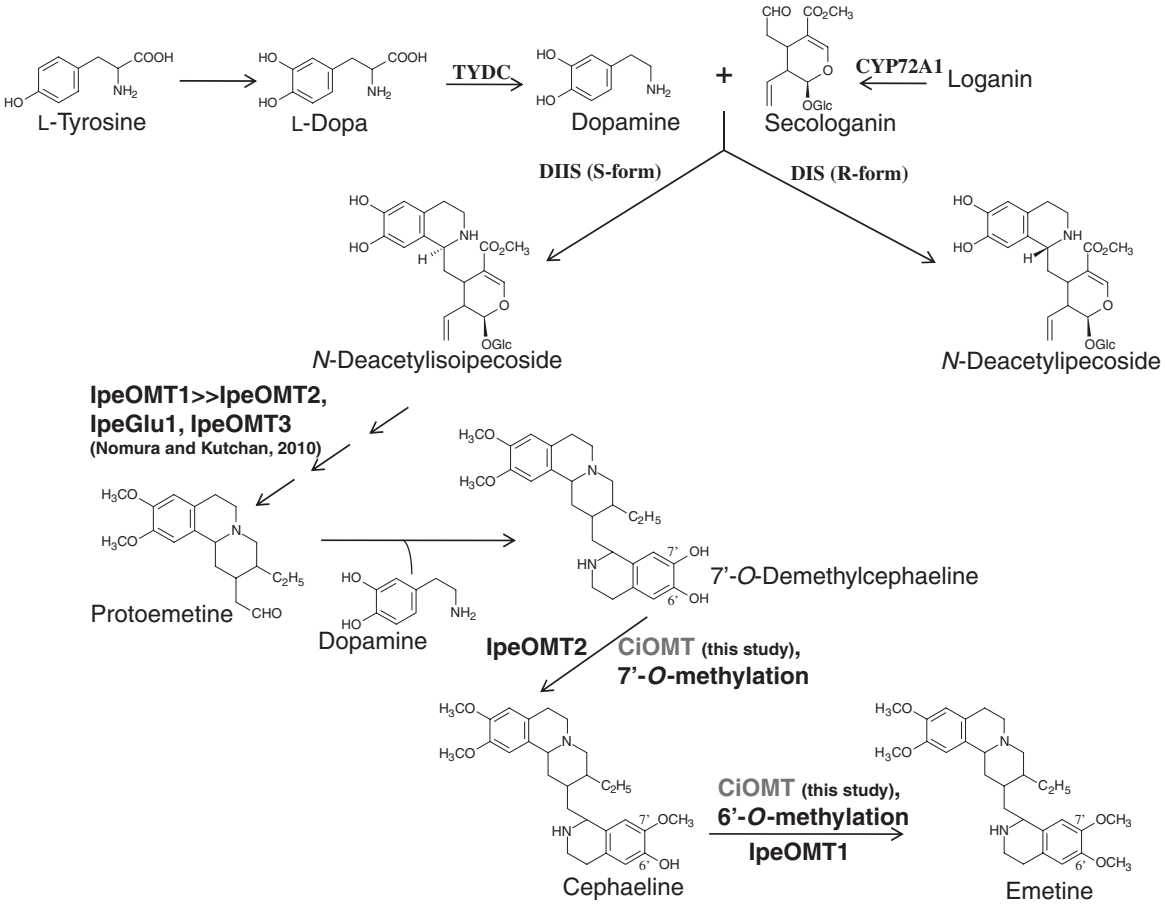

Fig. 1. Proposed Biosynthetic Pathway for Terpenoid Isoquinoline Alkaloids and Ipecac Alkaloids with an *S*-Configuration Derived from *N*-Deacetylisoipecoside (modified from Nomura and Kutchan, 2010).

TYDC, tyrosine/dopa decarboxylase; CYP72A1/SLS, secologanin synthase, DIS, deacetylipecoside synthase; DIIS, deacetylisoipecoside synthase.

Materials and Methods

Plant materials. Adventitious root cultures of ipecac were maintained as described previously.[10] In brief, liquid cultures were maintained in Murashige-Skoog medium[11] (pH 5.7) containing 3% sucrose and 1.0 mg/L of naphthalene acetic acid (NAA) in the dark at 25 °C at 4-week intervals at 100 rpm. Solid cultures were maintained with 0.25% Gellan gum with 0.5 mg/L of NAA at 12-week intervals.

Chemicals. Authentic cephaeline, emetine, and protoemetine were provided by K.Y. (Research Center for Medical Plant Resources, Tsukuba). Dopamine was purchased from Nacalai Tesque (Kyoto, Japan).

Isolation of O*-methyltransferase (OMT) cDNA from cultured ipecac adventitious roots.* Total RNAs were prepared from young (21-d-old) adventitious roots using Qiagen RNeasy Kits, following the manufacturer's instructions (Qiagen, Tokyo, Japan). cDNAs were then synthesized with oligo (dT) primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Three degenerate primers were designed based on the amino acid sequences of the OMTs of *Coffea arabica* (same plant family), *Coptis japonica* 6-OMT, and 4′-OMT (both in isoquinoline alkaloid biosynthesis, accession nos; D29811 and D29812). Fw1-1 (5′-GTNGAYGTNGGNGGNGGNA-CNGG-3′) and Fw1-2 (5′-GAYGTNGGNGGNGGNACNGGNAC-3′) were designed based on the conserved region motif A of most OMTs,[12] whereas Rv1 (5′-TCRTCNBWCCARTCRTGNARDAT-3′) was designed based on the region after motif B (where M = A or C, N = A, T, G or C, Y = C or T, R = A or G, K = T or G, W = A or T, S = C or G, D = A, G or T).

In the first PCR, the Fw1-1 and Oligo dT primers were used to amplify the fragments of ipecac OMT cDNAs under the following conditions: denaturation for 5 min at 94 °C, followed by 35 cycles of amplification with denaturation of 30 s at 94 °C, hybridization for 1 min at 50 °C, and extension for 1.20 min at 72 °C. Amplified products of about 400 bp to 650 bp were used in the second PCR using the Fw1-1, Fw1-2, and Rv1 primers under the same PCR conditions as in the first PCR. The resulting PCR products, of about 200 bp, were subcloned into pT7Blue T-vector (Novagen, Darmstadt, Germany) and sequenced. A clone (216 bp) showed a homologous sequence with OMT cDNA, and full-length cDNA cloning was conducted.

5′- and 3′-RACE were performed to obtain the 5′- and 3′-termini of the isolated OMT fragment following Sambrook *et al.*[13] Two gene-specific primers were designed for 3′-RACE: 3′-GSP1 (5′-CAGGGCCATTGCAAAAGCTTTCCCC-3′) and 3′-GSP1 nested (5′-GCTTTCCCCAACTTAAAGTGTACCG-3′). The resulting nested PCR products at about 650–800 bp were subcloned into pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) and their nucleotide sequences were determined completely.

Based on the nucleotide sequence obtained (690 bp), two gene-specific primers were further designed for 5′-RACE: 5′-GPS1 (5′-ATATTCATTACCAGCATTTTTCTCGAGTTC-3′) and 5′-GSP1 nested (5′-ACAATTCTCATCACTCCAGTCATGCAGAAC-3′). After the addition to the 3′ end of the template cDNA with oligo(dC) by terminal deoxynucleotidyl transferase (Promega), the 5′ end of the cDNA was amplified with primer Poly G (with adaptor) and 5′-GSP1, and then further nested. The resulting nested PCR products of about 800 bp for 5′-RACE were subcloned into pT7Blue T-vector, and the nucleotide sequences were determined completely. Based on the results of sequencing, the 5′ end obtained (807 bp) was combined with the previously obtained 3′ end sequence (600 bp) using BioEdit Sequence Alignment Editor Version 7.0 (http://www.mbio.ncsu.edu/

BioEdit/bioedit.html) to obtain the full-length sequence of the putative ipecac (Ci) OMT cDNA (1,258 bp).

Construction of expression vector for CiOMT1. Expression vector for CiOMT1 was constructed without the fused peptide derived from the vector sequence in a pET-41(a) vector (Novagen, Darmstadt, Germany). The 5′ end of the CiOMT1 sequence was amplified by PCR using the forward primer to introduce a *Nde*I site (5′-TGGCATATGGAAACAGTTCAG-3′) at the start codon, ATG. The 3′ end of the CiOMT1 sequence was amplified by PCR using the reverse primer (5′-CTAGCGTCGACTTCAAGGAGTAAGCTCGAT-3′) to introduce a *Sal*I site at the stop codon, TGA. pET-41(a) was digested with *Nde*I and *Sal*I, and the PCR products were ligated into the vector. These constructs (pECiOMT1) were sequenced to confirm that no change had been introduced during the subcloning process.

Heterologous expression of CiOMT1 in E. coli. The expression vector for CiOMT1 (pECiOMT1) was introduced into *E. coli* BL21 (DE3) cells. Expression of CiOMT1 was induced with 1 mM isopropylthiogalactoside (IPTG) at 16 °C for 24 h, and recombinant proteins were then extracted with extraction buffer (0.1 M Tris–HCl, pH 7.5, 10 mM sodium ascorbate, 10% glycerol, and 20 mM 2-mercaptoethanol). The extract was centrifuged at 14,000 rpm (about 18,000 × *g*) at 4 °C for 10 min, and then desalted through a NAP-5 column (GE Healthcare, Buckinghamshire, UK) for enzyme assay.

Assay of enzymatic activity. CiOMT1 activities were measured by HPLC and liquid chromatography-mass spectroscopy (LC-MS) after the enzyme reaction. The standard CiOMT1 reaction mixture consisted of 0.1 M potassium phosphate (Kpi, pH 6.8), 0.25 mM cephaeline, 0.5 mM AdoMet, 25 mM sodium ascorbate, and the enzyme preparation (600 µg). The assay mixture was incubated at 30 °C for 2 h, and incubation was terminated with the same volume of 90% methanol and 4% trichloroacetic acid (TCA). After protein precipitation, the reaction product was analyzed by reversed-phase HPLC (mobile phase, 30% acetonitrile containing 0.05% trifluoroacetic acid, TFA; column, TSKgel ODS-80TM (4.6 × 250 mm, TOSOH, Tokyo, Japan); flow rate, 0.5 mL/min; temperature of the column, 40 °C; detection, absorbance was measured at 280 nm with a SPD-10Avp photodiode array detector (Shimazu, Kyoto, Japan). The analysis conditions were the same for LC-MS (LCMS2010, Shimazu, Kyoto, Japan) except that absorbance was measured at 280 nm with a UV detector.

Assay of substrate specificity. To examine the substrate specificity of CiOMT1, 7′-*O*-demethylcephaeline, a putative precursor of cephaeline, was prepared by condensation of dopamine and protoemetine, as follows; twenty mM dopamine and 4 mM protoemetine were mixed in 100 mM Kpi buffer (pH 6.8) containing 25 mM sodium ascorbate in an Eppendorf tube under an N_2 atmosphere and kept tightly contained for 5 d in the dark at room temperature. The reaction mixture was diluted with 4 volumes of MilliQ water. The product was confirmed to be 7′-*O*-demethylcephaeline with *m*/*z* 453 by LC-MS. It was used in the enzyme assay.

In the assays of substrate specificity, the LC-MS analytical condition, *viz.*, the mobile phase, was changed to 20% acetonitrile containing 0.05% TFA at a flow rate of 0.7 mL/min.

Purification of recombinant CiOMT1 from E. coli *lysate.* Recombinant CiOMT1 was produced in 500 mL cultures of *E. coli* and extracted in the above-mentioned buffer containing 10 mM 2-mercaptoethanol and 10% glycerol. All procedures were performed at 4 °C. The crude bacterial extract was applied to a DEAE Fast Flow column (14.5 × 3 cm; Amersham Biosciences, Uppsala, Sweden) that had been equilibrated with 100 mM Tris–HCl (pH 7.5) containing 10% glycerol, 25 mM ascorbate, and 10 mM 2-mercaptoethanol. Proteins were eluted with a linear NaCl gradient of 0–1 M in 100 mM Tris–HCl (pH 7.5) (total volume, 160 mL) and enzyme activities were measured. Active fractions were pooled and kept at −80 °C with 40% glycerol until use.

The subunit molecular mass of the enzyme was analyzed by SDS–PAGE (12.5%), and the protein concentration was determined following Bradford,[14] using bovine serum albumin as standard.

Results

Isolation of OMT cDNA from ipecac

When we started this study, no OMT sequence in ipecac was available. Hence, we tried to isolate OMT cDNA from an adventitious ipecac root culture, which showed high alkaloid production,[10] using a homology-based cloning approach. For this purpose, we designed degenerate primers based on the conserved sequences for OMTs in isoquinoline alkaloid biosynthesis from *Coptis japonica*, *viz.*, 6-OMT and 4′-OMT,[9] and that of *Coffea arabica* (Rubiaceae, the same family as ipecac). Using these degenerate primers, we isolated a partial OMT cDNA (216 bp), that showed 76% identity with 16-hydroxytabersonine *O*-methyltransferase in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Cr16-OMT).[15] The full-length cDNA of OMT was then further isolated by 5′- and 3′-RACE.

Nucleotide sequence and predicted amino acid sequence

The isolated full-length cDNA of ipecac OMT (called CiOMT1; the nucleotide sequence has been deposited in the DDBJ/Genbank database under accession no. AB576187) contained 1,258 bp with an ORF that encoded 350 amino acids and showed high similarity to *Cr*16-OMT (52%), but lower to *C. arabica* OMT (42%), *C. japonica* 6-OMT (35%), and *C. japonica* 4′-OMT (34%). When the sequence of CiOMT1 was compared to those of ipecac OMTs that had been isolated recently,[5] CiOMT1 clearly showed very high identities with all three IpecOMTs. It showed 81%, 98%, and 63% identity with IpeOMT1, IpecOMT2, and IpeOMT3, respectively. IpeOMT2, with the highest identity, differed by only eight amino acids (Fig. 2A). Phylogenetic tree analysis (Fig. 2B) also indicated that CiOMT1 belongs to the same branch of ipecac OMTs and differs from those in isoquinoline alkaloid biosynthesis, such as *Cj*6′-OMT and *Cj*4-OMT. It also indicated a close relationship to *C. roseus* 16-OMT.[5]

Enzymological activity of the recombinant CiOMT1 produced in E. coli

To determine the enzymological activity of CiOMT1, an expression vector (pECiOMT1) was constructed, and recombinant protein was produced in *E. coli*. To avoid the effects of a tag-sequence, recombinant protein was produced as non-tagged polypeptides. The construct was then introduced into *E. coli* cells to induce production of the recombinant enzyme. Since recombinant CiOMT1 protein was produced in soluble form in large quantities (Fig. 3 lane A), the crude *E. coli* lysate was used to determine the enzymatic activity of CiOMT1.

When recombinant CiOMT1 was reacted with cephaeline as substrate, it showed clear *O*-methylation activity to produce emetine as IpeOMT1, whereas CiOMT1 showed the highest similarity to IpeOMT2 (the substrate specificities of CiOMT1 and IpeOMTs are discussed below). The formation of emetine was confirmed by LC-MS by direct comparison with authentic emetine, whereas the crude extract of *E. coli* carrying the heat-denatured enzyme showed no enzymatic activity (Fig. 4).

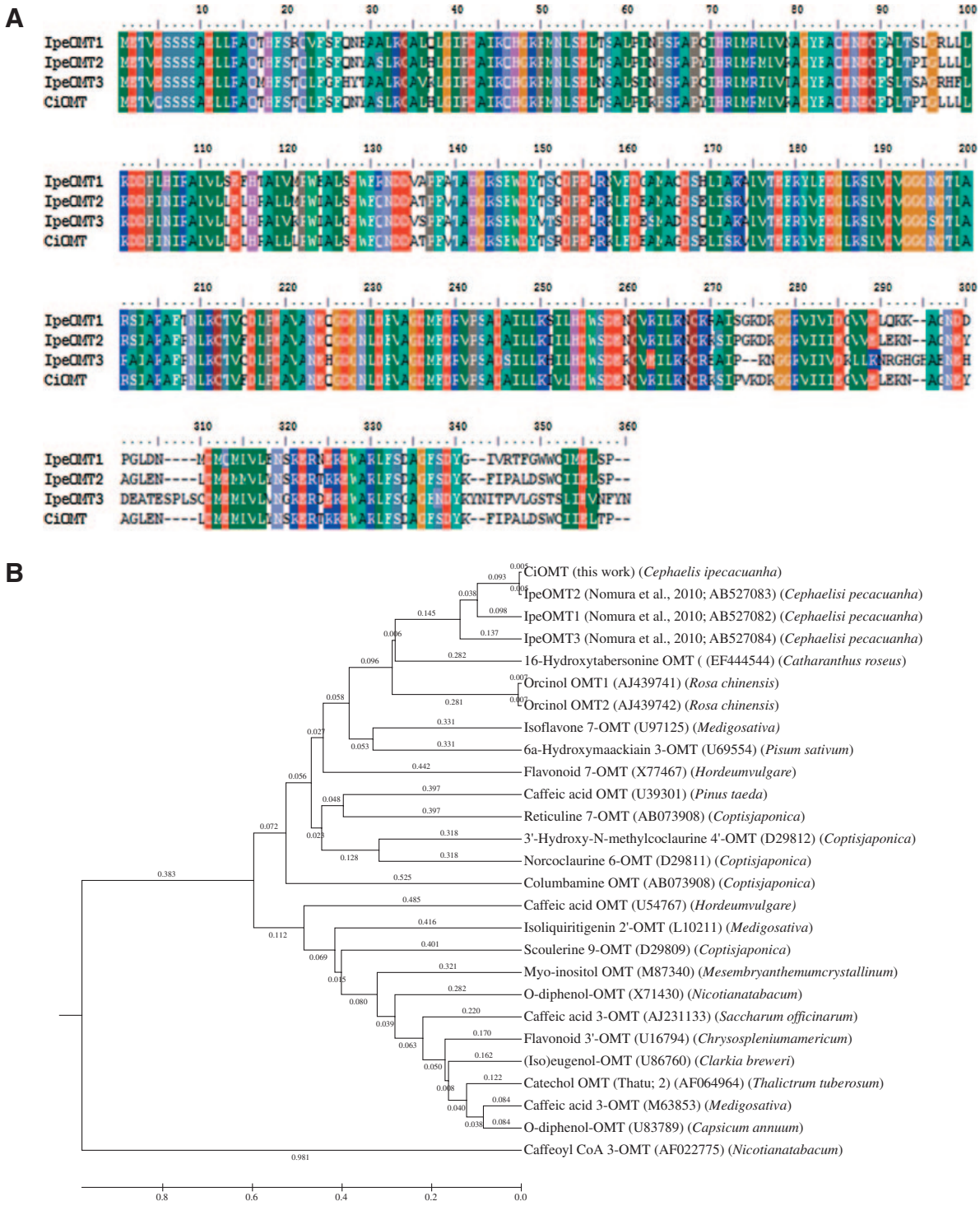

Fig. 2. Sequence Alignment and Phylogenetic Tree Analysis of CiOMT1.

(A) The amino acid sequence of the isolated CiOMT1 (this study) was compared with the three IpeOMTs (accession nos. AB527082–AB527084) reported by Nomura and Kutchan (2010).[5] (B) Phylogenetic tree of plant *S*-adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferase sequences. The OMT protein sequences were obtained from GenBank™ and used for tree-building. Overall, 27 sequences were aligned by the multisequence alignment program in MEGA 4.0.2 using the UPGMA (unweighted pair group maximum average) method.

Purification of recombinant CiOMT1 and characterization of it

Although CiOMT1 was confirmed to have *O*-methylation activity for cephaeline, it was relatively low. Hence, we tried to purify CiOMT1 by DEAE-Sepharose column chromatography. Whereas recombinant CiOMT1 was purified almost to homogeneity at approximately 40 kDa (SDS–PAGE, Fig. 3, lane B), the fold-purification was only 1.92 and recovery of enzyme activity was 14.8%, which indicates that CiOMT1 is unstable. Hence, we estimated the amount of CiOMT1 in the crude lysate using purified CiOMT1 as a reference. The amount of CiOMT1 in 2 µg of crude extract (Fig. 3) was estimated to be about 1.9 µg by ImageJ software (http://rsb.info.nih.gov/ij), and the crude extract was used directly in further enzymological characterization without purification.

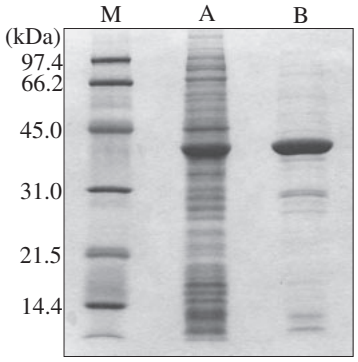

Fig. 3. SDS–PAGE of Crude and Purified Recombinant CiOMT1 from BL21 (λDE3) *E. coli* Cells.

Proteins were separated on 12.5% SDS–PAGE and stained with Coomassie Brilliant Blue G250. Lane M, molecular size marker; lane A, crude extract (10 μg/lane); lane B, DEAE Sepharose-purified enzyme (5 μg/lane).

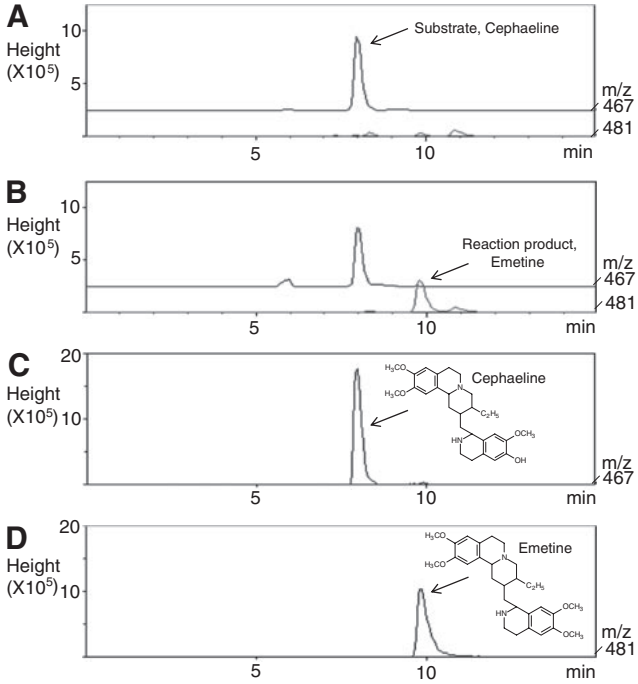

Fig. 4. *O*-Methylation Activity of CiOMT1 against Cephaeline.

(A) Reaction at 0 min (heat-denatured control). (B) Reaction for 2 h. Production of emetine was confirmed by both HPLC and LC-MS using authentic standards, cephaeline (C) and emetine (D) respectively. The substrate concentration of cephaeline was 500 μM.

Enzymological characterization of CiOMT1

Before the substrate specificity was characterized further, several enzymological properties were determined. The optimum pH (about pH 6.8) of CiOMT1 for the methylation of cephaeline was similar to that of IpeOMT2 for *N*-deacetylisoipecoside (pH 7.0), but much lower than that of IpeOMT1 for *N*-deacetylisoipecoside (pH 8.0)[5] (Nomura and Kutchan, 2010) or those of the OMTs in isoquinoline alkaloid biosynthesis in *Coptis japonica*.[9,16] On the other hand, CiOMT1 did not require divalent cations for its activity, and the addition of EDTA, Ca^{2+}, and Mg^{2+} individually had no effect on CiOMT1 activity, whereas the addition of Co^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+} individually at 5 mM strongly inhibited CiOMT1 activity, by 53%, 83%, 90%, and 31% respectively. Enzyme activity was also negligibly inhibited by iodoacetamide (an SH reagent).

Substrate specificity and affinity of CiOMT1

Since CiOMT1 showed the highest homology to IpeOMT2, which *O*-methylates 7′-*O*-demethylcephaeline, the OMT activity of CiOMT1 for substrates other than cephaeline was examined. Our preliminary experiments with crude root extract as substrate suggested that CiOMT1 has other OMT activity (data not shown). However, since we lacked a suitable substrate, we tried to prepare a substrate, 7′-*O*-demethylcephaeline, by spontaneous condensation of dopamine and protoemetine, as described above in "Experimental Procedures." LC-MS analysis confirmed formation of the m/z 453 product, and this mass value was identical to that of 7′-*O*-demethylcephaeline (Fig. 5A).

Incubation of CiOMT1 with the substrates prepared from dopamine and protoemetine showed the formation of cephaeline, indicating that prepared 7′-*O*-demethylcephaeline (m/z 453) was successfully converted to cephaeline (m/z 467) by CiOMT1 after 30 min of reaction (Fig. 5B). CiOMT1 further methylated cephaeline to produce emetine after 24 h of reaction (Fig. 5F). This clearly indicates that CiOMT1 has broad regional OMT activity for both 7′-*O*-demethylcephaeline and cephaeline.

Nomura and Kutchan[5] have reported that the Km value of IpecOMT2 for 7′-*O*-demethylcephaeline (1.0 μM) was much lower than that of IpecOMT1 for cephaeline. To examine the preference of CiOMT1 for these substrates, we conducted a competitive assay with two substrates at 10 μM, since we did not have sufficient 7′-*O*-demethylcephaeline. As shown in Fig. 5, CiOMT1 predominantly used 7′-*O*-demethylcephaeline as a substrate to produce cephaeline; almost all of the 7′-*O*-demethylcephaeline was converted to cephaeline by CiOMT1 within 2 min. The amount of cephaeline increased rapidly. On the other hand, emetine, the methylation product of cephaeline, was not observed, which indicates that CiOMT1 did not use cephaeline as a preferred substrate as compared to 7′-*O*-demethylcephaeline (Fig. 5B). When all of the 7′-*O*-demethylcephaeline was converted to cephaeline, after 10 min, emetine was gradually produced from cephaeline by CiOMT1.

The kinetic parameters of CiOMT1 for a substrate were also determined for comparison to those of IpeOMTs, whereas only those for cephaeline were determined, due to a lack of substrate availability. CiOMT1 showed Michaelis-Menten-type reaction kinetics for cephaeline and double-reciprocal plots of the initial velocity *versus* the substrate concentration with various concentrations of cephaeline (0.03–1 mM) and AdoMet (0.125–4 mM). The apparent Km values for cephaeline and AdoMet were 68 ± 23 μM and 97 ± 35 μM respectively, with a Vmax of approximately 0.3 nmol min^{-1} mg^{-1} (Table 1). The Km and Vmax values of CiOMT1 for cephaeline were similar to those of IpeOMT1, but the Km of IpeOMT2 for 7′-*O*-demethylcephaeline was much smaller, and the Vmax was greater (Fig. 6).[5] Characterization of the kinetic parameters of CiOMT1 for 7′-*O*-demethylcephaeline should help in the effort to understand the molecular mechanism of these three enzymes with very similar amino acid sequences.

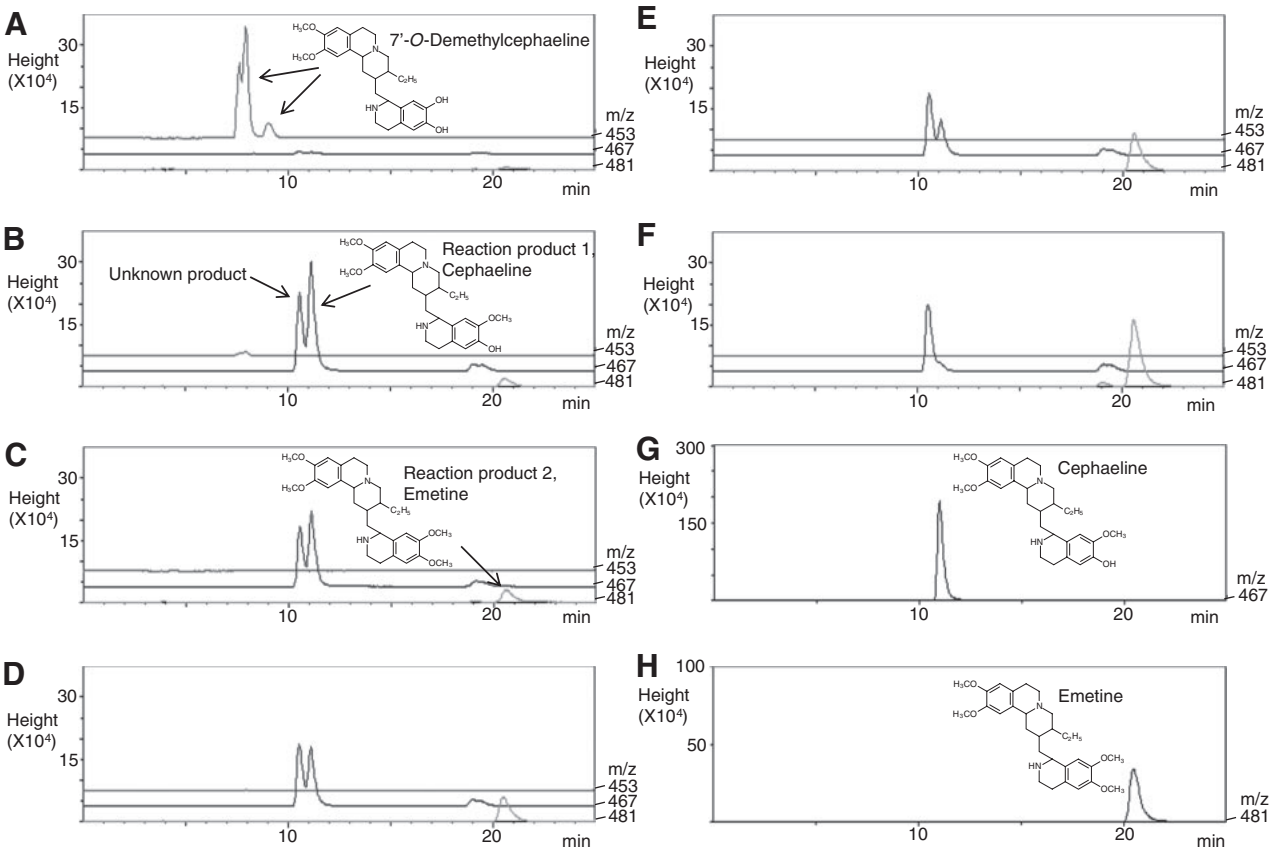

Fig. 5. Broad Regional Enzyme Activity of CiOMT1.

CiOMT1 sequentially methylated both 7′-*O*-demethylcephaeline and cephaeline to produce cephaeline and emetine respectively. A mixture of dopamine and protoemetine was reacted with CiOMT1 (A). Reaction at 0 min. (B)–(F) CiOMT1 reaction for 30 min, 1 h, 2 h, 4 h, and 24 h respectively. (G)–(H) Authentic cephaeline and emetine respectively. Reaction products 1 and 2 showed the same retention times and m/z values as authentic cephaeline and emetine respectively.

Table 1. Comparison of Kinetic Parameters of OMTs Isolated from Ipecac

Enzyme (Ref.)	Substrate	Km (μM)	Vmax (nmol min^{-1} mg^{-1})	Optimum pH
IpeOMT1	Cephaeline	80 ± 7	0.41 ± 0.01	8.0
(Nomura and Kutchan, 2010)	AdoMet	42 ± 6		(*N*-Deacety-isoipecoside as substrate)
IpeOMT2	7′-*O*-Demethyl-cephaeline	1.0 ± 0.5	47.6 ± 2.7	7.0
(Nomura and Kutchan, 2010)	AdoMet	20 ± 1		(*N*-Deactyl-isoipecoside as substrate)
CiOMT1	Cephaeline	68 ± 23	0.34 ± 0.01	6.8
(This study)	AdoMet	97 ± 35		(Cephaeline as substrate)

Discussion

O-Methyltransferases (OMTs) play important roles in the biosynthesis of secondary metabolites, including ipecac alkaloids. Recently, Nomura and Kutchan[5] reported the isolation of three IpeOMTs, each of which had a different enzymological role in biosynthesis. IpeOMT2 catalyzes the 7′-*O*-methylation of 7′-*O*-demethylcephaeline to form cephaeline, and IpeOMT1 shows 6′-*O*-methylation activity toward cephaeline to form emetine at the last two steps of emetine biosynthesis, whereas the endogenous substrate for IpeOMT3 is not known. While these IpeOMTs showed broad substrate specificities toward the intermediates in emetine biosynthesis, their regional substrate specificities at the last steps of emetine biosynthesis were restricted to 7′-*O*-methylation or 6′-*O*-methylation (Fig. 1). Our CiOMT1 showed both the activity of IpeOMT2 and that of IpeOMT1, and it might play a role at the last two steps (Fig. 1). Although we could not determine which OMT is actually involved at the last two steps, our analysis of the kinetic properties suggests that these enzymes have comparable enzymatic activity. Our data, as well as the existence of many isoenzymes reported by Nomura and Kutchan (AB556938–AB556943),[5] suggest that the OMTs in ipecac alkaloid biosynthesis are highly redundant. Further careful investigation of the expression of each gene is needed, while Nomura and Kutchan suggested that both IpeOMT1 and IpeOMT2 are involved at the last two steps.

While this redundancy might be due to natural variations in the materials used, further studies are needed to determine the physiological importance of these OMTs. We are currently working to design more specific primers to measure the expression level of each OMT quantitatively in different tissues and cells in comparison with alkaloid biosynthesis, while our preliminary analysis with rather common primers suggested

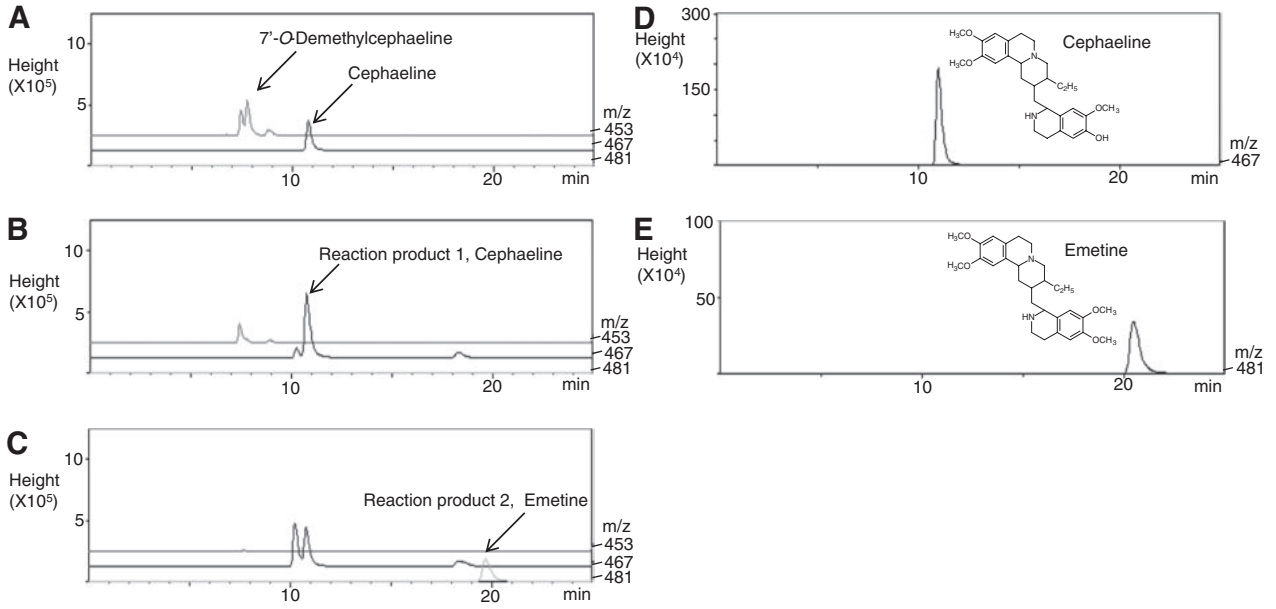

Fig. 6. Competitive Reaction of CiOMT1 for 7′-*O*-Demethylcephaeline and Cephaeline.
(A)–(C) 7′-*O*-Demethylcephaeline and cephaeline were reacted with CiOMT1 for 0 min, 2 min, and 2 h. Reaction products 1 and 2 showed the same retention times and m/z values as authentic cephaeline (D) and emetine (E) respectively.

that there is a good correlation between the expression of OMTs and alkaloid biosynthesis (data not shown).

In addition to providing new insight into the physiological importance of OMTs in alkaloid biosynthesis, CiOMT1 provides unique molecular information about reaction specificity, since IpeOMT2, which has only an 8-amino acid substitution, showed a different substrate specificity and different kinetic properties. IpeOMT2 *O*-methylates 7′-*O*-demethylcephaeline but not cephaeline, whereas CiOMT1 *O*-methylates both. While some OMTs in isoquinoline alkaloid biosynthesis have been reported to have strict substrate specificity and reaction specificity,[9] a recent re-investigation of some enzymes showed that 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) not only contributes to 4′-*O*-methylation but also to the 6-*O*-methylation of norcoclaurine.[17] Furthermore, characterization of a chimeric enzyme of norcoclaurine 6-*O*-methyltransferase and 4′-OMT showed that their substrate specificity and reaction specificity can be modified by substitution in the N-terminal region, which might be involved in substrate binding.[18] IpeOMT2 has glutamic acid, asparagine, and methionine at positions 5, 63, and 121 respectively, while CiOMT1 has glutamine, lysine, and leucine at these positions. These differences in three amino acids at the N-terminal ends of IpeOMT2 and CiOMT1 can provide clues to understand their reaction specificities. Successful expression of recombinant CiOMT1 in soluble form should provide sufficient material for the crystallographic characterization of CiOMT1 in the future, and this should help to clarify the unique specificity not only of the enzyme itself but also of other plant OMTs involved in secondary metabolite biosynthesis.

In addition, these three OMTs (IpeOMT1, IpeOMT2, and CiOMT1) are also interesting materials for understanding the evolution of these genes in ipecac alkaloid biosynthesis. Whereas 6-*O*-methylation is predominant in the biosynthesis of most isoquinoline alkaloids, 7-*O*-methylation (7′-*O*-methylation) has been found to be predominant at the last two steps of emetine biosynthesis in ipecac, which are catalyzed by these three unique OMTs. Since this is a rare example of OMT activity in isoquinoline alkaloid biosynthesis, it would be interesting to determine how these three genes evolved. Direct sequencing of the genomic DNA of ipecac plants should provide useful information.

References

1) Trease GE and Evans WC, "Pharmacognosy" 13th edn, Bailliere Tindal, London, pp. 595–599 (1989).
2) Yoshimatsu K and Shimomura K, "Biotechnology in Agriculture and Forestry, Vol. 21, Medicinal and Aromatic Plants IV," ed. Bajaj YPS, Springer, Berlin, pp. 87–103 (1993).
3) Sato F, Inui T, and Takemura T, *Curr. Pharmaceut. Biotechnol.*, **8**, 211–218 (2007).
4) Nomura T, Quesada AL, and Kutchan TM, *J. Biol. Chem.*, **283**, 34650–34659 (2008).
5) Nomura T and Kutchan TM, *J. Biol. Chem.*, **285**, 7722–7738 (2010).
6) De-Eknamkul W, Suttipanta NT, and Kutchan TM, *Phytochemistry*, **55**, 177–181 (2000).
7) Zubieta C, He XZ, Dixon RA, and Noel JP, *Nat. Struct. Biol.*, **8**, 271–279 (2001).
8) Minami H, Kim JS, Ikezawa N, Takemura T, Katayama T, Kumagai H, and Sato F, *Proc. Natl. Acad. Sci. USA*, **105**, 7393–7398 (2008).
9) Morishige T, Tsujita T, Yamada Y, and Sato F, *J. Biol. Chem.*, **275**, 23398–23405 (2000).
10) Yoshimatsu K and Shimomura K, *Phytochem. Anal.*, **4**, 217–219 (1993).
11) Murashige T and Skoog F, *Physiol. Plant.*, **15**, 473–497 (1962).
12) Joshi CP and Chiang VL, *Plant Mol. Biol.*, **37**, 663–674 (1998).
13) Sambrook J, Fritsch EF, and Maniatis T, "Molecular Cloning, a Laboratory Manual" 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 8.54–8.61 (1989).
14) Bradford MM, *Anal. Biochem.*, **72**, 248–254 (1976).
15) Levac D, Murata J, Kim WS, and de Luca V, *Plant J.*, **53**, 225–236 (2008).
16) Takeshita N, Fujiwara H, Mimura H, Fitchen JH, Yamada Y, and Sato F, *Plant Cell Physiol.*, **36**, 29–36 (1995).
17) Inui T, Tamura K, Fujii N, Morishige T, and Sato F, *Plant Cell Physiol.*, **48**, 252–262 (2007).
18) Morishige T, Tamakoshi M, Takemura T, and Sato F, *Proc. Jpn. Acad., Ser. B*, **86**, 757–768 (2010).