Three New O-Methyltransferases Are Sufficient for All O-Methylation Reactions of Ipecac Alkaloid Biosynthesis in Root Culture of Psychotria ipecacuanha
Taiji Nomura1, 2, and Toni M. Kutchan1
From 1Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132, USA
2Present address: Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan
Running head: Ipecac alkaloid O-methyltransferases
Address correspondence to: Taiji Nomura, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan. Tel: 81-766-56-7500 (ex. 517); Fax: 81-766-56-2498; E-mail: tnomura@pu-toyama.ac.jp

The medicinal plant Psychotria ipecacuanha produces Ipecac alkaloids, a series of monoterprenoid-isoquinoline alkaloids such as emetine and cephaeline, whose biosynthesis derives from condensation of dopamine and secologanin. Here, we identified three cDNAs, IpeOMT1-IpeOMT3, encoding Ipecac alkaloid O-methyltransferases (OMTs) from P. ipecacuanha. They were coordinately transcribed with the recently identified Ipecac alkaloid β-glucosidase IpeGlu1. Their amino acid sequences were closely related to each other, and rather to the flavonoid OMTs than to the OMTs involved in benzylisoquinoline alkaloid biosynthesis. Characterization of the recombinant IpeOMT enzymes with integration of the enzymatic properties of the IpeGlu1 revealed that emetine biosynthesis branches off from N-deacetylisopecoside through its 6-O-methylation by IpeOMT1, with a minor contribution by IpeOMT2, followed by deglucosylation by IpeGlu1. The 7-hydroxy group of the isoquinoline skeleton of the aglycon is methylated by IpeOMT3 prior to the formation of protoemetine that is condensed with a second dopamine molecule, followed by sequential O-methylations by IpeOMT2 and IpeOMT1 to form cephaeline and emetine, respectively. In addition to this central pathway of Ipecac alkaloid biosynthesis, formation of all methyl derivatives of Ipecac alkaloids in P. ipecacuanha could be explained by the enzymatic activities of IpeOMT1-IpeOMT3, indicating that they are sufficient for all O-methylation reactions of Ipecac alkaloid biosynthesis.

Plants produce a diverse array of secondary metabolites, whose number has been estimated to be over 200,000. In plants a large portion of those natural products are considered to be synthesized for chemical defense against microbial attack and herbivore predation because of their biological activities. Since some of them exhibit a wide range of pharmacological activities, they have also been studied from the aspect of pharmaceutical sources. Alkaloids are one of the better studied classes of plant natural products due to their pharmacological activities. Psychotria ipecacuanha Stokes (Rubiaceae), which is native to South and Central America, has been used in therapy since the early 17th century as an emetic and expectorant, and later as medication for amebic dysentery (1). Such medicinal effects of the root extracts derive from the principal alkaloid emetine. P. ipecacuanha also produces many kinds of emetine-related alkaloids such as cephaeline and ipecoside, known as Ipecac alkaloids (2) (Fig. 1). Alangium lamarckii Thwaites (Alangiaceae), a medicinal plant indigenous to India (2), also produces Ipecac alkaloids.

Structurally, Ipecac alkaloids possess a monoterprenoid-isoquinoline skeleton that originates from secologanin, a glucosidal monoterprenoid, and dopamine. Secologanin and dopamine are condensed in a Pictet-Spengler manner to form the 1α(S)-diastereomer N-deacetylisopecoside and the 1β(R)-diastereomer N-deacetylipepecoside (Fig. 1). The reaction proceeds non-enzymatically under weakly acidic conditions, but it was demonstrated that two enzymes, each of which stereo-specifically catalyzes the formation of each diastereomer, are involved in the reaction in Alangium (3, 4). The 1(S)-diastereomer N-deacetylisopecoside is deglucosylated and converted to protoemetine, which is then condensed with a second molecule of dopamine and further converted to cephaeline and emetine (Fig. 1). The 1(R)-diastereomer...
Dopamine is analogous to that of moiety of Ipecac alkaloids that derives from expected that modification of the isoquinoline terpenoid-indole alkaloid pathway. It is reactions, the Ipecac alkaloid pathway is similar to condensation and the following deglucosylation (Fig. 4 in ref. 15). With respect to the first culture that catalyzes the deglucosylation of the \( \beta \)-glucosidase, IpeGlu1 accepts not only the \( \beta \)-configured glucoalkaloids function in defense when toxic ipecoside aglycon is released by IpeGlu1 in response to pathogen and herbivore attack (15).

Although \( N \)-deacetylipecoside aglycon is highly reactive and is converted into multiple forms through non-enzymatic intra-molecular reactions in vitro, we have proposed that the iminium cation formed by dehydration is the intermediate for emetine biosynthesis in vivo (see Fig. 4 in ref. 15). With respect to the first condensation and the following deglucosylation reactions, the Ipecac alkaloid pathway is similar to the terpenoid-indole alkaloid pathway. It is expected that modification of the isoquinoline moiety of Ipecac alkaloids that derives from dopamine is analogous to that of benzylisoquinoline alkaloids, the biosynthesis of which begins with the condensation of dopamine and 4-hydroxyphenylethylacetaldehyde. Two hydroxy groups of the isoquinoline skeleton are methylated by \( S \)-adenosyl-L-methionine (SAM)-dependent \( O \)-methyltransferases (OMTs). Several OMTs catalyzing these reactions have been identified in opium poppy Papaver somniferum, Coptis japonica, Thalictrum tuberosum and Eschscholzia californica: norcoclaveine 6-OMT (16-18), reticuline 7-OMT (16), norreticuline 7-OMT (19), scoulerine 9-OMT (20) and columbamine 2-OMT (21). In addition to these OMTs, 3'-hydroxy-N-methylcoclaurine 4'-OMT (22), coclaurine \( N \)-methyltransferase (23) and stylopine \( cis \)-N-methyltransferase (24) catalyze \( O \)- and \( N \)-methyltransfer reactions specific to benzylisoquinoline alkaloid biosynthesis.

Ipecac alkaloids possess one isoquinoline moiety in the carbon skeleton prior to condensation with the second molecule of dopamine to form cephaeline and emetine, which contain two isoquinoline groups (Fig. 1). At completion of emetine biosynthesis, four hydroxy groups are methylated. Two hydroxy groups of the first isoquinoline skeleton are methylated during protoemetine formation. IpeGlu1 is involved in the deglucosylation step, but it is unknown whether \( O \)-methylations occur before or after deglucosylation (Fig. 1). In addition, \( O \)-methylolation reactions of the second isoquinoline skeleton to form the final product emetine have not been determined. Moreover, a large part of the chemical diversity of Ipecac alkaloids is attributable to the distinct \( O \)-methylation patterns with respect to the four hydroxy moieties. Thus, OMTs are essential targets to be identified for the elucidation of the order of transformations occurring along the Ipecac alkaloid biosynthetic pathway. In the present study, we report the identification of three cDNAs encoding Ipecac alkaloid OMTs in P. ipecacuanha through EST analysis coupled with the characterization of the substrate specificity and kinetic parameters of recombinant enzymes. The substrate specificities of the three recombinant OMTs are sufficient to explain all \( O \)-methylation reactions of Ipecac alkaloid biosynthesis. Based on their functional characterization, we propose the detailed biosynthetic network of Ipecac alkaloids in P. ipecacuanha.
EXPERIMENTAL PROCEDURES

**Plant Materials**— *P. ipecacuanha* root cultures were maintained as described by ref. 15.

**Cloning of O-Methyltransferase cDNAs**— 1050 ESTs from a λ-ZAP cDNA library constructed from mRNA of *P. ipecacuanha* in vitro roots were recently sequenced (15). Homology searches of the ESTs using the BLASTX algorithm revealed that one of the contigs (contig-213) was similar to known plant OMTs. The contig consisted of four EST members (ph1903-35, ph1903-62, ph1803-II-61 and ph3011-41), of which ph1903-62 and ph1803-II-61 were identical in the overlapped region, but were different from ph1903-35 and ph3011-41. Oligonucleotide primers were designed from common sequences among them for the isolation of full-length coding sequences by RT-PCR; 5’-AACTTGGCAAATGGAAACTG-3’ (forward) and 5’-CTTGAATTCAAGGAGAAAGCTC-3’ (reverse). Root mRNA was purified as described in ref. 15 and the cDNA synthesized using a SMART RACE cDNA Amplification Kit (Clontech) was used for the PCR: 30 sec at 98°C, followed by 35 cycles of amplification (10 sec at 98°C, 30 sec at 55°C, and 1 min at 72°C) in a 50-μl reaction mixture containing 3 ng of cDNA, 0.5 μM primers, 0.2 mM dNTPs, 1 x reaction buffer and 1 U of Phusion HS-HF polymerase (Finnzymes). The PCR products were inserted into pCR-BluntII-TOPO vector (Invitrogen) and sequenced to obtain *IpeOMT1* and *IpeOMT2*. *IpeOMT1* corresponded to the EST ph3011-41, and *IpeOMT2* to the ph1903-62 and ph1803-II-61. Because the full-length cDNA clone corresponding to the EST ph1903-35 was not obtained, 3’-RACE was performed using ph1903-35-specific forward primer (5’-GTCTAGATTAATAGCATCATAAGTTAG G-3’) and the adapter primer under the same conditions as described above except for the annealing temperature at 60°C. The PCR products were inserted into pCR-BluntII-TOPO and sequenced to obtain *IpeOMT3*, which corresponded to the EST ph1903-35.

**Heterologous Expression and Purification of IpeOMT Enzymes**— The entire coding region of each of the three *IpeOMT* s was amplified by PCR and ligated in between the *NdeI* and *EcoRI* restriction sites of a pET28a vector (Novagen). The resulting vector was introduced into the *E. coli* strain BL21 CodonPlus(DE3) RIL (Stratagene) for protein expression. The recombinant *E. coli* was grown in 500 ml of LB medium containing 30 μg/ml kanamycin and 35 μg/ml chloramphenicol at 37°C until the OD600 reached 0.6-0.8. After cooling, IPTG was added at 1 mM and the culture was incubated for 18 h at 18°C on an orbital shaker at 200 rpm. The cells were harvested (6000 x g, 10 min) and resuspended in 20 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 20% (v/v) glycerol, 10 mM 2-mercaptoethanol and 0.5% (v/v) protease inhibitor cocktail (Sigma). After sonication (20 sec at 50 W, 6 times) and centrifugation (20000 x g, 20 min), the supernatant containing soluble protein was collected for the purification of the N-terminal His-tagged IpeOMT proteins.

The recombinant protein was purified with TALON His-Tag Purification Resin (Clontech). All operations were done at 4°C. The resin (1 ml of bed volume per 20 ml of the supernatant) equilibrated in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M KCl, 20% glycerol and 10 mM 2-mercaptoethanol was mixed with the supernatant for 1 h. After the resin was washed with equilibration buffer, the recombinant IpeOMT protein was eluted in a stepwise manner by increasing imidazole concentration (5 mM and 200 mM) in equilibration buffer (5 ml for each elution). The 200 mM imidazole fraction was subjected to a PD10 column (GE Healthcare) equilibrated with storage buffer (50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl, 20% glycerol and 5 mM dithiothreitol), and was concentrated appropriately by ultrafiltration using a Centriprep YM-10 (Millipore). Purified protein was stored at -80°C.

**Substrates for Enzyme Assays**— Of the 52 compounds listed in supplemental Fig. S1, ipecoside, cephaeline, 21 benzylisoquinoline alkaloids, kaempferol and myricetin were from the chemical stock of Dr. Meinhart H. Zenk (Donald Danforth Plant Science Center). Quercetin and resveratrol were gifts from Dr. Oliver Yu (Donald Danforth Plant Science Center).
7'-O-Demethylcephaeline was gift from Dr. Takao Tanahashi (Kobe Pharmaceutical University, Japan). Dopamine, 3-hydroxy-4-methoxyphenethylamine, 4-hydroxy-3-methoxyphenethylamine, caffeic acid, guaiacol, catechol, vanillin, vanillic acid and isovanillic acid were purchased from Sigma and Acros Organics. N-deacetylisoipecoside, N-deacetylisoipecoside, 6-O-methyl-N-deacetylisoipecoside, 6-O-methyl-N-deacetylisoipecoside, 7-O-methyl-N-deacylisoipecoside, 7-O-methyl-N-deacetylisoipecoside, demethylisoalangiside, and demethylalangiside were prepared according to ref. 15. Isoalangiside, 3'-O-demethylisoalangiside, and 3'-O-demethyl-2'-O-methylalangiside were synthesized from 6-O-methyl-N-deacetylisoipecoside, 6-O-methyl-N-deacetylisoipecoside, 7-O-methyl-N-deacetylisoipecoside, and 7-O-methyl-N-deacetylisoipecoside, respectively, according to the method for synthesis of lactams (15).

Redipecamine (reduced Ipecac amine, designated in the present study) was prepared by deglucosylating N-deacetylisoipecoside with Ipecac alkaloid β-glucosidase (IpeGlu) under reducing conditions (15). The deglucosylation reaction was performed in 2 ml of 0.1 M citrate/0.2 M phosphate buffer (pH 5.0) containing 10 mM N-deacetylisoipecoside, 10 mM NaBH₃CN and 108 µg of IpeGlu9 enzyme. After 1-h incubation at 55°C, the reaction was stopped by adding 400 µl of 1 N HCl, and the denatured enzyme was removed by centrifugation (16000 x g, 2 min). To the supernatant was added 400 µl of 10% (w/v) Na₂CO₃, followed by the extraction with ethyl acetate (2.8 ml x 2). The organic layer was dried under N₂ flow, and the residue was dissolved in 1 ml of 50% (v/v) methanol containing 0.1% (v/v) TFA and subjected to preparative HPLC (column, LiChrosorb RP18, 25 x 250 mm, 7 µm, Merck; eluent, A = 0.1% (v/v) TFA, B = acetonitrile, gradient = 10 - 40% (v/v) B/(A+B) in 50 min; flow rate, 8 ml/min; detection, 230 nm). The collected fraction was concentrated in vacuo and freeze-dried.

3-O-Methylredipecamine was prepared from N-deacetylisoipecoside via 6-O-methyl-N-deacetylisoipecoside. The reaction mixture (30 ml) containing 0.1 M citrate/0.2 M phosphate buffer (pH 6.5), 1 mM N-deacetylisoipecoside, 10 mM SAM and 4.6 mg of IpeOMT1 enzyme was incubated at 40°C. After 7-h incubation, SAM (65 mg) and 0.9 mg of IpeOMT1 enzyme were added and further incubated for 2 h. The reaction was stopped by adding 6.2 ml of 1 N HCl, and the precipitation formed was removed by filtration (0.2 µm, Whatman), and the pH was adjusted to 5.0 with 0.2 M Na₂HPO₄. To the solution (55 ml) was added 1.8 ml of 10 mM NaBH₃CN dissolved in 0.1 M citrate/0.2 M phosphate buffer (pH 5.0) and 360 µg of IpeGlu9 enzyme. After 1-h incubation at 55°C, the reaction was stopped by adding 12 ml of 1 N HCl. The pH was adjusted to 9.0 with 10% Na₂CO₃, and ethyl acetate extraction was performed (100 ml x 3). The organic layer was washed with saturated NaCl solution and evaporated in vacuo to dryness. The residue was dissolved in 1 ml of acetonitrile containing 0.1% TFA, subjected to preparative HPLC as described above, and the collected fraction was concentrated in vacuo and freeze-dried. 2-O-Methylredipecamine was prepared from 7-O-methyl-N-deacetylisoipecoside using the same procedure.

Enzyme Assay- In a series of screening of substrates for the IpeOMT1, IpeOMT2 and IpeOMT3 enzymes, standard enzyme reaction was performed in 0.1 M KPi buffer (pH 7.5) containing 10 µg of enzyme, 1 mM SAM and 1 mM substrate (varied between 0.05-1 mM with solubility and availability) in a total volume of 100 µl. After incubation at 30°C for 2 h, the reaction was terminated by the addition of 20 µl of 1 N HCl. After centrifugation (16000 x g, 2 min), the supernatant was subjected to HPLC analysis to detect the reaction products (column, Nova-Pak C18, 3.9 x 300 mm, 4 µm, Waters; eluent, A = 0.1% TFA, B = acetonitrile, gradient = 12 - 80% B/(A+B) in 30 min; flow rate, 0.8 ml/min; detection, 230 nm). For the analysis of dopamine, 3-hydroxy-4-methoxyphenethylamine, 4-hydroxy-3-methoxyphenethylamine, guaiacol, and catechol, conditions for UV detection and solvent gradient were changed appropriately.

For the determination of pH optimum, the same buffer systems were used as described in ref.
15. Temperature optimum was determined by the reactions at 25-55°C. N-deacetylisopecoside was used as substrate for IpeOMT1 and IpeOMT2, and (S)-reticuline for IpeOMT3.

Enzyme reactions for the determination of kinetic parameters were performed in 0.1 M KPi buffer at the optimum pH for each IpeOMT at 30°C. Kinetic parameters were calculated from three replicates by non-linear fitting of the data to the Michaelis-Menten equation using SigmaPlot 2001.

Identification of Enzymatic Products- The enzymatic products that are dependent on the presence of SAM were identified by comparing the retention time and the fragmentation pattern in Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis with authentic compounds. For LC-MS/MS analysis, HPLC separation was conducted with a Shimadzu LC20A system, and mass spectra were obtained using a 4000 Q-TRAP triple stage quadrupole mass spectrometer equipped with a TurboionSpray ionization source (Applied Biosystems) in a positive ion mode. The HPLC separation was performed under the following conditions: (column, Nova-Pack C18, 3.9 x 300 mm, 4 μm, Waters; eluent, solvent A = 0.2% formic acid, solvent B = acetonitrile; flow rate, 0.8 ml/min). Solvent gradient conditions basically followed that described above, but were changed appropriately when the 6-O-methylate and 7-O-methylate of isoquinolines needed to be separated. For the analyses of IpeOMT1 and IpeOMT2 reaction mixtures with cephaeline and 7'-O-demethylcephaeline to detect emetine and cephaeline, respectively, the column was changed to Lichrospher 60 RP-select B (4 x 250 mm, 5 μm, Merck) for better peak shape of those substrates and products. To identify the molecular ion of the reaction products, MS detection was done by enhanced mass scan (collision energy, 10 V; declustering potential, 60 V), and then the fragmentation pattern of the molecular ion detected was analyzed by enhanced product ion scan, for which the declustering potential was kept at 80 V while the collision energy was adjusted appropriately.

To identify the enzymatic reaction products of N-deacetylisopecoside by IpeOMT1 and IpeOMT2, the products were derivatized with di-tert-butyldicarbonate ((Boc)₂O) before LC-MS/MS analysis, because 6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside could not be separated by HPLC under any conditions tested. For the analysis of IpeOMT1 reaction product, the enzyme reaction was performed in 0.1 M KPi (pH 7.5) containing 1.7 mM N-deacetylisopecoside, 2 mM SAM and 200 μg of IpeOMT1 enzyme in a total volume of 1 ml. After 4-h incubation at 30°C, the reaction was terminated by addition of 0.2 ml of 1 N HCl, followed by centrifugation (16000 x g, 2 min). The supernatant was subjected to preparative HPLC under the same conditions as described above except for the gradient condition (10 - 21% B/(A+B) in 60 min). The collected fraction was evaporated in vacuo and freeze-dried. The residue was treated with 1 μmol of (Boc)₂O in methanol containing 1 μmol of triethylamine for 1 h at room temperature. The reaction mixture was dried under N₂ flow, and dissolved in 500 μl of 50% methanol. The chromatographic behavior of the N-Boc derivative was compared with those of standard 6-O-methyl-N-Boc-deacetylisopecoside and 7-O-methyl-N-Boc-deacetylisopecoside synthesized according to ref. 15. To identify the enzymatic reaction products of N-deacetylisopecoside by IpeOMT2, the enzyme reaction was performed in 0.1 M citrate/0.2 M phosphate buffer (pH 6.5) containing 0.5 mM N-deacetylisopecoside, 1 mM SAM, 1 mM EDTA, 1 mg/ml BSA and 400 μg of IpeOMT2. After the overnight incubation at 40°C, the reaction was terminated by 0.2 ml of 1 N HCl. Purification and N-Boc derivatization of the products were conducted in the same procedure as described above, and subjected to LC-MS/MS analysis.

Subcellular Localization of IpeOMTs and IpeGlu1- The coding sequences of IpeOMT1, IpeOMT2, IpeOMT3 and IpeGlu1 (GenBank accession number AB455576) were amplified by PCR and inserted into pBA103 vector (gift from Brian Kelly, Donald Danforth Plant Science Center), which possesses an enhanced cauliflower mosaic virus 35S promoter, an enhanced green fluorescence protein (eGFP) gene and a nopaline synthase terminator, to express C-terminal GFP fused proteins, IpeOMT1-GFP, IpeOMT2-GFP, IpeOMT3-GFP and IpeGlul1-GFP. DNA-coated gold particles (0.6 mm, Bio-Rad) were prepared according to the manufacturer’s instructions, and...
bombarded into a small piece (approximately 2 cm x 2 cm) of onion with a PDS1000 He Biolistic Particle Delivery System (Bio-Rad) according to manufacturer’s instructions. The bombarded onion was wrapped with a wet paper towel, and incubated at room temperature overnight in a petri dish. Cytosol- and vacuole-targeted enhanced yellow fluorescence protein (eYFP) constructs (gift from Dr. Isabel Ordiz, Donald Danforth Plant Science Center), endoplasmic reticulum (ER)-targeted eGFP construct (gift from Dr. Nigel Taylor, Donald Danforth Plant Science Center), and pBA103 without insert were also bombarded as controls. After the incubation, the epidermis was peeled off and analyzed using a LSM 510 Confocal/Multiphoton microscope (Zeiss). Fluorescence of GFP and YFP was visualized by excitation at 488 nm and a band-pass emission filter (505-550 nm). Confocal images were analyzed using the Imaris software (Bitplane).

**Isolation of O-Methyltransferase cDNAs from P. ipecacuanha**- RT-PCR from mRNA of P. ipecacuanha root culture resulted in the isolation of two full-length cDNAs IpeOMT1 (Ipecac alkaloid O-methyltransferase 1, GenBank accession number AB527082) and IpeOMT2 (AB527083). IpeOMT1 corresponded to the EST ph3011-41, and IpeOMT2 to the ph1903-62 and ph1803-II-61. The 3'-RACE PCR specific to the EST ph1903-35 resulted in the isolation of IpeOMT3 (AB527084).

IpeOMT1, IpeOMT2 and IpeOMT3 encoded polypeptides of 350, 350 and 358 amino acids with calculated molecular masses of 38.7, 39.0 and 39.7 kDa, respectively. The deduced amino acid sequences were highly similar to each other, where IpeOMT1 showed 82% and 72% identities to IpeOMT2 and IpeOMT3, respectively, and IpeOMT2 showed 73% identity to IpeOMT3. IpeOMT1, IpeOMT2 and IpeOMT3 amino acid sequences exhibited the highest identity (55%, 59%, and 55%, respectively) to the flavonoid OMTs of C. roseus (27), which catalyzes O-methylation of 3'- and 5'-hydroxy groups of myricetin and dihydromyricetin. IpeOMT3 also shared comparable identity (53%) to resveratrol OMT that catalyzes methylations of 3- and 5-hydroxy groups of resveratrol to form pterostilbene in Vitis vinifera (28). Phylogenetic analysis of the IpeOMTs with selected members of plant OMTs, including benzylisoquinoline alkaloid OMTs (Fig. 2) showed that three IpeOMTs are closely related to each other and the clade exhibited the closest relationship to the flavonoid OMTs and terpenoid-indole alkaloid 16-hydroxytabersonine 16-OMT in C. roseus (29), but not to the OMTs of benzylisoquinoline alkaloids such as 3'-hydroxy-N-methylcoclaurine 4'-OMT in C. japonica (22), norcoclaurine 6-OMTs in P. somniferum (16), T. tuberosum (18) and C. japonica (22), reticuline 7-OMT (16), norreticuline 7-OMT (19), columbamine 2-OMT (21), and scoulerine 9-OMT (20).

**Quantitative RT-PCR Analysis**- P. ipecacuanha in vitro roots precultured for eight weeks were transferred to new LS liquid medium (26), and the roots were harvested in duplicate 1 day, 3 days, 1 week, 2 weeks, 4 weeks, 6 weeks, and 8 weeks after transfer. Total RNA was purified using an RNeasy Plant Mini Kit (Qiagen), followed by DNase treatment and re-purification. The synthesis of cDNA was performed with a SuperScript III Reverse Transcriptase (Invitrogen) (0.5 µg of RNA in 20 µl of reaction mixture). Duplicates were further pooled and 5 ng of cDNA was used for PCR. PCR was performed in triplicate on a StepOnePlus Real-Time PCR system (Applied Biosystems) under the following conditions: 5 min at 95°C, followed by 40 cycles of amplification (15 sec at 95°C, and 1 min at 60°C) in a 20-µl reaction mixture containing template cDNA, 0.5 µM primers, and 10 µl of PerfeCTa SYBR Green FastMix Reaction Mixes (Quanta Biosciences). Primers specific to each of IpeOMT1, IpeOMT2, IpeOMT3 and Ipeglu1 were used (supplemental Table S1). A standard curve was made for every reaction with the dilution series of the known quantities of a plasmid having each of the target cDNA as template. Specificity of the amplification was verified by a melt-curve analysis at the end of each PCR.

**RESULTS**
tested with seven glucosidal Ipecac alkaloids (Table 1; substrates 1-7, supplemental Fig. S1), including N-deacetylisopecoside, N-deacetylisopecoside and their 6- and 7-O-monomethylates. In the reaction of IpeOMT1 using N-deacetylisopecoside as substrate, product dependent upon the presence of IpeOMT1 and the methyl donor SAM was detected by HPLC analysis. Since the possible products 6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside could not be separated by reverse-phase HPLC under conditions tested (Fig. 4A), the product was purified and chemically converted to the N-tert-butoxycarbonyl (N-Boc) derivative. LC-MS/MS profiles of the derivative were the same as those of authentic 6-O-methyl-N-Boc-deacetylisopecoside (Fig. 4B), showing that IpeOMT1 catalyzed 6-O-methylation of N-deacetylisopecoside regio-specifically to form 6-O-methyl-N-deacetylisopecoside. IpeOMT2 also reacted with N-deacetylisopecoside and a SAM-dependent products were detected (Fig. 4A). Product identification was performed after N-Boc derivatization. Two peaks were detected and they showed the same LC-MS/MS profiles as authentic 6-O-methyl-N-Boc-deacetylisopecoside and 7-O-methyl-N-Boc-deacetylisopecoside (Fig. 4B). Thus, it was found that IpeOMT2 catalyzed both a 6- and 7-O-monomethylation of N-deacetylisopecoside, where 6-OMT activity was approximately three times higher than 7-OMT activity, as determined from the chromatogram of the N-Boc derivatives (Fig. 4B). In contrast to IpeOMT1 enzyme that showed no activity towards 1(R)-diastereomer N-deacetylisopecoside, IpeOMT2 reacted with N-deactetylisopecoside, as well as 1(S)-diastereomer N-deacetylisopecoside. By LC-MS/MS analysis the products were identified to be 6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside (Fig. 4C). In the reaction, 7-O-methylation occurred approximately eight times more efficiently than 6-O-methylation. In addition to the substrate and the products, their lactams formed by non-enzymatic intra-molecular reaction were also detected (Fig. 4C). The enzyme assay with O-monomethylates of N-deacetylisopecoside and N-deacetylisopecoside revealed that IpeOMT1 also catalyzed 6-O-methylation of 7-O-methyl-N-deacetylisopecoside to form 6,7-O,8-dimethyl-N-deacetylisopecoside (Fig. 4D).

Enzymatic activities for lactam substrates (Table 1; substrates 8-13, supplemental Fig. S1) were also tested. IpeOMT1 did not show any activity for the lactam substrates. IpeOMT2 catalyzed O-methylation of the 2-hydroxy group of demethylalangiside, which corresponds to the 7-hydroxy group with respect to the isoquinoline skeleton, to form 3-O-demethyl-2-O-methylalangiside (Fig. 4E). Its activity was regio- and stereo-selective, in contrast to the activities for non-lactam glucosidal Ipecac alkaloids as described above. IpeOMT3 accepted none of the 13 glucosidal Ipecac alkaloids (Table 1; substrates 1-13) tested as substrate.

To examine the involvement of IpeOMT enzymes in 6’- and 7’-O-methylation of the second isoquinoline moiety in emetine biosynthesis (Fig. 1), their activities toward 7’-O-demethylcephaeline, the penultimate intermediate for emetine biosynthesis, and toward cephaeline (Table 1; substrates 14 and 15, supplemental Fig. S1) were tested. As a result, IpeOMT2 methylated the 7’-hydroxy group of 7’-O-demethylcephaeline to form cephaeline (Fig. 4F), and IpeOMT1 methylated the 6’-hydroxy group of cephaeline to form emetine (Fig. 4G). IpeOMT3 was active neither with 7’-O-demethylcephaeline nor cephaeline.

Although an endogenous substrate for IpeOMT3 was not known at this stage, since (S)-reticuline, one of the benzylisoquinoline alkaloids, was found to serve as its substrate as described below, pH and temperature optima were determined using N-deacetylisopecoside as substrate for IpeOMT1 and IpeOMT2 and (S)-reticuline for IpeOMT3. The pH optimum of IpeOMT1 was 8.0, whereas those of IpeOMT2 and IpeOMT3 were 7.0. Temperature optima of IpeOMT1, IpeOMT2 and IpeOMT3 were 40°C, 45°C and 40°C, respectively.

**Substrate Specificity of the IpeOMT Enzymes**- To reveal structural feature of substrates for each IpeOMT enzyme, enzyme reactions were performed using various substrates, including benzylisoquinoline alkaloids (Table 1; substrates 19-39, supplemental Fig. S1) and simple phenolics (substrates 40-48, supplemental Fig. S1). Simple phenolics were not accepted as substrate by the
IpeOMT1, IpeOMT2 and IpeOMT3 enzymes. IpeOMT1 catalyzed 6-O-methylation of (R,S)-isococlaurine, (R,S)-norcooclaurine, (S)-4’-O-methyllaudanosoline, (R,S)-isoorientaline, (R)-norprotosinomenine, (S)-norprotosinomenine, and (R,S)-protosinomenine, and an exceptional 4’-O-methylation of (R,S)-nororientaline (Table 1). The results indicated that IpeOMT1 dominantly catalyzes 6-O-methylation of the 6,7-dihydroxy- and 6-hydroxy-7-methoxy isoquinolines. This is consistent with the IpeOMT1 activities for Ipecac alkaloids as described above (substances 1, 6 and 15).

IpeOMT2 catalyzed 6-O-methylation of (R,S)-isococlaurine, (R,S)-norcooclaurine and (R,S)-isoorientaline (Table 1), showing that IpeOMT2 catalyzes 6-O-methylation of 6,7-dihydroxy- and 6-hydroxy-7-methoxy isoquinolines. This is different from the IpeOMT2 activities for Ipecac alkaloids described above, i.e. 6-O-monomethylation and 7-O-monomethylation activities toward the 6,7-dihydroxy isoquinolone moiety. Considering that 6- and 7-O-methylation of N-deacetylisoipecoside was catalyzed only by IpeOMT2 and that 7-O-methyl-N-deacetylisoppecoside was synthesized only by IpeOMT2 (Figs. 7 and 8), this relaxed regio-specificity appears to be an essential feature in Ipecac alkaloid biosynthesis. It is notable, however, that IpeOMT2 regio-specifically catalyzed the methylation of the 7’-hydroxy group, but not the 6’-hydroxy group, of 7’-O-demethylcephaeline to form cephaeline, which is considered to be the primary reaction of IpeOMT2 judging from the catalytic parameters (Table 2). Although 3-OMT activity toward oripavine was detected, since 3-hydroxy group of oripavine is not bound to an isoquinoline skeleton, this activity seems to be fortuitous.

IpeOMT3 catalyzed the 7-O-methylation of (S)-cooclaurine, (R,S)-N-methylcooclaurine, (R,S)-4’-O-methylcooclaurine, (R,S)-6-O-methyllaudanosoline, (R,S)-nororientaline, (S)-norreticuline, and (S)-reticuline (Table 1). IpeOMT3 accepted only 7-hydroxy-6-methoxy isoquinolines. Although the methylated position of the enzymatic product of coreximine could not be assigned due to the lack of authentic 2-O-methylcoreximine and 11-O-methylcoreximine, and possibly due their identical fragmentation pattern in MS/MS analysis, methylation of the 2-hydroxy group that corresponds to 7-hydroxy group of isoquinoline skeleton is most likely, judging from the regio-specificity of IpeOMT3 enzyme for other benzylisoquinoline alkaloids.

Although IpeOMT3 was found to catalyze 7-O-methylation of 7-hydroxy-6-methoxy benzylisoquinoline alkaloids, they are not the endogenous substrates, because none of these benzylisoquinoline alkaloids have been found in *P. ipecacuanha*. However, the results encouraged us to examine the Ipecac alkaloid aglycon as a possible endogenous substrate. As shown in ref. 15, deglucosylation of N-deacetylisoipecoside gives multiple products formed by non-enzymatic intra-molecular reactions of the highly reactive aglycon. Redipecamine (supplemental Fig. S1) that is formed by the deglucosylation reaction under reducing conditions could, however, be stably prepared (15). Therefore, we used redipecamine, 2-O-methylredipecamine and 3-O-methylredipecamine (Table 1; substrates 16-18, supplemental Fig. S1) as mimics of endogenous Ipecac alkaloid aglycons. IpeOMT1 reacted with none of them. IpeOMT2 methylated the 2-hydroxy group of redipecamine (Fig. 4H) that corresponds to the 7-hydroxy group of isoquinoline skeleton, while IpeOMT3 catalyzed 2-O-methylation of 3-O-methylredipecamine (Fig. 4H). These results strongly suggested that IpeOMT2 and IpeOMT3 accept endogenous Ipecac alkaloid aglycons that are formed by deglucosylation of N-deacetylisoipecoside and 6-O-methyl-N-deacetylisoipecoside, respectively.

Since IpeOMT sequences exhibited the highest identities to the flavonoid OMT of *C. roseus*, and IpeOMT3 also exhibited comparably high identity to the resveratrol OMT of *V. vinifera*, IpeOMT activities toward kaempferol, quercetin, myricetin, and resveratrol (substrates 49-52, supplemental Fig. S1) were examined, but none were accepted by IpeOMT as substrate.

Kinetic parameters of the IpeOMT enzymes for Ipecac alkaloids, synthetic mimics of the Ipecac alkaloid aglycons, and the methyl donor SAM were determined (Table 2). Apparent *K_m* and apparent *k_cat* values of IpeOMT1 for *N*-deacetylisoipecoside were 107 μM and 11.3 x 10^3 s^−1, respectively. The apparent *K_m* value for 7-O-methyl-N-deacetylisoipecoside (105 μM) was equal to that for *N*-deacetylisoipecoside, but the
The apparent \( k_{\text{cat}} \) value for 7-O-methyl-\( N \)-deacetylisoipecoside (20.4 \( \times 10^{-3} \) s\(^{-1}\)) was 2-fold higher than that for \( N \)-deacetylisoipecoside. The catalytic efficiency of IpeOMT1 for cephaeline (3.5 s\(^{-1}\)M\(^{-1}\)) was remarkably lower than those for \( N \)-deacetylisoipecoside (106 s\(^{-1}\)M\(^{-1}\)) and 7-O-methyl-\( N \)-deacetylisoipecoside (194 s\(^{-1}\)M\(^{-1}\)) mainly due to the lower \( k_{\text{cat}} \) (0.28 \( \times 10^{-3} \) s\(^{-1}\)) in spite of the comparable \( K_m \) (80 \( \mu \)M).

Kinetic parameters of IpeOMT2 for \( N \)-deacetylisoipecoside were evaluated based on the total amount of the two products, 6-O-methyl-\( N \)-deacetylisoipecoside and 7-O-methyl-\( N \)-deacetylisoipecoside, because they could not be separated by HPLC analysis. The apparent reaction efficiency (0.81 s\(^{-1}\)M\(^{-1}\)) was approximately 130-fold lower than that of IpeOMT1 (106 s\(^{-1}\)M\(^{-1}\)) due to larger \( K_m \) (420 \( \mu \)M) and lower \( k_{\text{cat}} \) (0.34 \( \times 10^{-3} \) s\(^{-1}\)) values. Kinetic parameters of IpeOMT2 for \( N \)-deacetylisoipecoside were not determinable, because considerable amounts of substrate and the enzymatic products (6-O-methyl-\( N \)-deacetylisoipecoside and 7-O-methyl-\( N \)-deacetylisoipecoside) were non-enzymatically converted to their lactams under the reaction conditions used (pH 7.0) (see Fig. 4C). The apparent reaction efficiency for demethylalangiside (8.4 s\(^{-1}\)M\(^{-1}\)) was 10-fold higher than that for \( N \)-deacetylisoipecoside due to the 10-fold increase of the \( k_{\text{cat}} \) value (3.8 \( \times 10^{-3} \) s\(^{-1}\)) with a similar \( K_m \) value (450 \( \mu \)M). IpeOMT2 catalyzed the 2-\( O \)-methylation of the synthetic \( N \)-deacetylisoipecoside aglycon mimic, redipecamine, with greater reaction efficiency (66 s\(^{-1}\)M\(^{-1}\)) than those for \( N \)-deacetylisoipecoside and demethylalangiside. The apparent reaction efficiency of IpeOMT2 for 7'-O-demethylcephaeline (32600 s\(^{-1}\)M\(^{-1}\)) was the highest among the substrates tested, which is attributable to notably small \( K_m \) (1.0 \( \mu \)M) and high \( k_{\text{cat}} \) (32.6 \( \times 10^{-3} \) s\(^{-1}\)) values.

The apparent \( K_m \) (64 \( \mu \)M) of IpeOMT3 for 3-O-methylredipecamine, the synthetic 6-O-methyl-\( N \)-deacetylisoipecoside aglycon mimic, was 2-fold larger and the apparent \( k_{\text{cat}} \) (10.5 \( \times 10^{-3} \) s\(^{-1}\)) was 5-fold higher than those of IpeOMT2 for redipecamine.

Kinetic parameters of IpeOMT1, IpeOMT2 and IpeOMT3 for the methyl donor SAM, were also determined using \( N \)-deacetylisoipecoside (for IpeOMT1 and IpeOMT2) and (S)-reticuline (for IpeOMT3) as methyl acceptors. The apparent \( K_m \) values of IpeOMT1, IpeOMT2 and IpeOMT3 were comparable to each other (42 \( \mu \)M, 20 \( \mu \)M and 34 \( \mu \)M, respectively). Although the apparent \( k_{\text{cat}} \) values of IpeOMT1 and IpeOMT3 were similar (7.1 \( \times 10^{-3} \) s\(^{-1}\) and 8.9 \( \times 10^{-3} \) s\(^{-1}\), respectively), that of IpeOMT2 (0.26 \( \times 10^{-3} \) s\(^{-1}\)) was remarkably lower than those of IpeOMT1 and IpeOMT3.

**Subcellular Localization of IpeOMT and IpeGlu1 Enzymes** To examine the subcellular localization of IpeOMT1, IpeOMT2 and IpeOMT3 enzymes, C-terminal-GFP fused enzymes (IpeOMT1-GFP, IpeOMT2-GFP and IpeOMT3-GFP) were transiently expressed in onion epidermal cells. In addition, subcellular localization of the Ipecac alkaloid \( \beta \)-glucosidase (IpeGlu1) (15) was also examined by expressing IpeGlu1-GFP fusion protein to see whether or not IpeGlu1 enzyme is compartmentalized separately from the IpeOMT enzymes. Three-dimensional images were reconstructed from the confocal images, and the localization of GFP/YFP signals was analyzed. Fig. 5 shows the representative sections obtained from the transformed onion cells. Fluorescence profiles of onion cells expressing each of the IpeOMT-GFPs and IpeGlu1-GFP were the same as those expressing non-targeted GFP and cytosol-targeted YFP, but were totally different from those expressing ER-targeted GFP and vacuole-targeted YFP. These results showed the cytosolic localization of IpeOMT and IpeGlu1 enzymes. This was supported by the absence of signal peptides in IpeOMT and IpeGlu1 sequences as predicted by the computer programs iPSORT, SignalP and Target P.

**Transcript Profiles of IpeOMT and IpeGlu1 Genes in P. ipecacuanha in vitro Roots** Transcript profiles of the IpeOMT1, IpeOMT2 and IpeOMT3 genes, as well as the Ipeglu1 gene were analyzed in *P. ipecacuanha* root culture in order to see whether the three IpeOMT genes are coordinately transcribed with Ipeglu1. Time-course changes in the gene transcription in root cultures were measured by quantitative RT-PCR analysis (Fig. 6). Throughout the time-course, transcript patterns of the three IpeOMTs were well correlated with that of Ipeglu1, where transcript levels started to increase after...
subculture, reached maxima in 1-2 weeks, and then decreased to lower levels. The transcript level of IpeOMT3 was lower than the other two IpeOMTs and Ipeglu1.

DISCUSSION

Involvement of IpeOMTs in the Biosynthesis of I(S)-N-Deacetylisoipecoside- Identification of the three Ipecac alkaloid O-methyltransferases, IpeOMT1, IpeOMT2 and IpeOMT3, in the present study, as well as the Ipecac alkaloid β-glucosidase, Ipeglu1 (15) enabled us to elucidate a major portion of the biosynthetic pathways to Ipecac alkaloids. Although the EST database of Psychotria root culture that was constructed consisted of relatively small number of ESTs (1050 members) (15), the results indicate that it is enriched in Ipecac alkaloid biosynthetic genes.

The elucidated pathway and the contribution of each of those enzymes are shown in Figs. 7 and 8 for S- and R-forms of Ipecac alkaloids, respectively. Both IpeOMT1 and IpeOMT2 enzymes catalyzed the 6-O-methylation of N-deacetylisoipecoside, an intermediate in emetine biosynthesis. Kinetic analysis showed that the reaction catalyzed by IpeOMT1 is 130 times as efficient as that catalyzed by IpeOMT2 due to smaller $K_m$ and higher $k_{cat}$ values, indicating that IpeOMT1 plays a major role in the formation of 6-O-methyl-N-deacetylisoipecoside.

7-O-methylation of N-deacetylisoipecoside was catalyzed by IpeOMT2, although the reaction efficiency was much lower than the 6-O-methylation reaction catalyzed by IpeOMT1 and IpeOMT2. 6,7-O,O-dimethyl-N-deacetylisoipecoside was found to be synthesized via 7-O-methyl-N-deacetylisoipecoside, because none of the IpeOMT1s catalyzed 7-O-methylation of 6-O-methyl-N-deacetylisoipecoside but IpeOMT1 catalyzed the 6-O-methylation of 7-O-methyl-N-deacetylisoipecoside. These enzymatic properties seem to be the mechanism to effectively prepare 6-O-methyl-N-deacetylisoipecoside to be deglucosylated by Ipeglu1 for emetine biosynthesis. In fact, 6-O-methyl-N-deacetylisoipecoside is one of the best substrates for Ipeglu1 among the glucosidal Ipecac alkaloids having an S-configuration, while its activity was extremely poor toward 7-O-methyl-N-deacetylisoipecoside and 6,7-O,O-dimethyl-N-deacetylisoipecoside (15).

Deglucosylation of 6-O-methyl-N-deacetylisoipecoside by Ipeglu1 gives multiple products due to non-enzymatic intra-molecular reactions, of which the iminium cation formed by dehydration of the immediate Ipeglu1 product was supposed to serve as intermediate for emetine biosynthesis (15). In order to examine the involvement of IpeOMTs in the other O-methylation reaction (i.e. methylation of the 7-hydroxy group with respect to isoquinoline skeleton) prior to protoemetine formation, we tested synthetic 3-O-methylredipecamine (see supplemental Fig. S1) as a mimic of endogenous 6-O-methyl-N-deacetylisoipecoside aglycon. That resulted in the detection of 2-O-methylation activity of IpeOMT3 toward 3-O-methylredipecamine, which strongly supports the hypothesis that the methylation of the 7-hydroxy group of the isoquinoline skeleton occurs after degraduclorylation of 6-O-methyl-N-deacetylisoipecoside at a certain biosynthetic step leading to protoemetine (Fig. 7).

These results suggest that methylation of the 6-hydroxy group before the 7-hydroxy group of the isoquinoline skeleton is a common feature in the biosynthesis of isoquinoline alkaloids. In the biosynthesis of the benzylisoquinoline alkaloids papaverine, laudanine and palmatine, 6-hydroxy group of isoquinoline skeleton is methylated in the early step by norcoclaurine 6-OMT (16, 18, 22), and then the 7-hydroxy group is methylated in a later step by norreticuline 7-OMT (19), reticuline 7-OMT (16) and columbamine 2-OMT (21), respectively. In P. ipecacuanha, however, two sequential O-methylation steps leading from 7'-O-demethylcephaeline, the condensation product of protoemetine and the second dopamine, to emetine were performed in the opposite manner, where the 7'-hydroxy group of 7'-O-demethylcephaeline was methylated first by the highly specific ($K_m = 1.0 \, \mu M$) IpeOMT2 to form cephaeline, followed by 6'-O-methylation of cephaeline to form the end product emetine by IpeOMT1 (Fig. 7). The catalytic efficiency of IpeOMT1 toward cephaeline (3.5 s$^{-1}$·M$^{-1}$) was much lower than that of IpeOMT2 toward
7'-O-demethylcephaeline (32600 s⁻¹·M⁻¹). These enzymatic properties may be the reason for accumulation of both cephaeline and emetine as major Ipecac alkaloids in P. ipecacuanha (1) in spite of the expression of IpeOMT1 and IpeOMT2 at the comparable levels (Fig. 6).

In addition to this central biosynthetic pathway of Ipecac alkaloids, branch pathways were also elucidated. Since none of the IpeOMT enzymes accepted the lactam species demethylisoalangiside, isoalangiside, and 3-O-demethyl-2-O-methylisoalangiside as substrates, isoalangiside, 3-O-demethyl-2-O-methylisoalangiside and methylisoalangiside were presumed to be synthesized by spontaneous lactamization of corresponding methylated N-deacetylisopecoinoside as shown in Fig. 7. 9-O-demethylcephaeline and 10-O-demethylcephaeline have been isolated from Alangium (2), and 10-O-demethylcephaeline has also been found in Psychotria (30, Cephaelis in the literature). They are considered to be synthesized from 9-O-demethylprotoemetine and 10-O-demethylprotoemetine (Fig. 7). Considering that IpeGlu1 deglucosylates N-deacetylispecoinoside as efficiently as 6-O-methyl-N-deacetylispecoinoside (15) and that IpeOMT2 catalyzed 2-O-methylation of rediipecamine, the synthetic mimic of endogenous N-deacetylispecoinoside aglycon, 9-O-demethylprotoemetine is formed through the pathway shown in Fig. 7. It is plausible that a certain amount of N-deacetylispecoinoside is deglucosylated by IpeGlu1 prior to 6-O-methylation by IpeOMT1 and IpeOMT2. Likewise, 10-O-demethylprotoemetine can be formed from 6-O-methyl-N-deacetylispecoinoside aglycon. 9-O-demethylprotoemetine and 10-O-demethylprotoemetine are probably condensed with dopamine, and the products are further methylated by IpeOMT2 to form 9-O-demethylcephaeline and 10-O-demethylcephaeline (Fig. 7).

**Involvement of IpeOMTs in the Biosynthesis of Ipecac Alkaloids**

Ipecac alkaloids derived from 1(R)-N-deacetylispecoinoside are known to be accumulated as alkaloidal glucosides such as ipecoside and alangiside (5, 6). Their O-methyl derivatives (Fig. 8) have also been identified (2). It was found herein that O-methylation reactions of the alkaloidal glucosides having an R-configuration are catalyzed only by IpeOMT2 (Table 1), the catalytic property of which revealed the biosynthetic pathway to the R-form Ipecac alkaloids (Fig. 8). IpeOMT2 activity for 6-O-methylation and 7-O-methylation of N-deacetylispecoinoside and the absence of activity catalyzing 6-O-methylation and 7-O-methylation of ipecoside demonstrated that 6-O-methylispecoinoside and 7-O-methylispecoinoside are synthesized from 6-O-methyl-N-deacetylispecoinoside and 7-O-methyl-N-deacetylispecoinoside, respectively. Since none of the IpeOMTs catalyzed 3-O-methylation of demethylalangiside, alangiside is considered to be formed by spontaneous lactamization of 6-O-methyl-N-deacetylispecoinoside. Meanwhile, 3-O-demethyl-2-O-methylalangiside is synthesized not only by the lactamization of 7-O-methyl-N-deacetylispecoinoside, but also by the enzymatic 2-O-methylation of demethylalangiside by IpeOMT2. 6,7-O,6-dimethyl-N-deacetylispecoinoside and its lactam methylalangiside have not been found so far in P. ipecacuanha. This would be justified by the fact that none of the IpeOMTs catalyzed their formation (Fig. 8). However, we cannot exclude the possibility that other OMTs that were not discovered in the present study are involved in their formation, because methylalangiside has been isolated in Alangium (31, 32). Deep transcriptome sequencing would enable the complete identification of the remaining Ipecac alkaloid biosynthetic genes, including such OMT(s), as well as those involved in the unidentified pathway leading to protoemetine from 6-O-methyl-N-deacetylispecoinoside aglycon.

**Subcellular Network of Ipecac Alkaloid Biosynthetic Enzymes**

It has been demonstrated that the terpenoid-indole alkaloid, strictosidine, which is formed by condensation of tryptamine and secologanin, is synthesized and stored in the vacuole (33, 34). Likewise, in P. ipecacuanha N-deacetylispecoinoside and N-deacetylispecoinoside that are formed by the condensation of dopamine and secologanin should be synthesized in the vacuole. We demonstrated that IpeOMTs and IpeGlu1, which work toward N-deacetylispecoinoside and N-deacetylispecoinoside, are localized in cytosol (Fig. 5). This means that N-deacetylispecoinoside and N-deacetylispecoinoside need to be transported outside the vacuole to react
with the subsequent enzymes. For emetine biosynthesis, \(N\)-deacylsoipecoside transported into the cytosol immediately has to be captured by IpeOMT1 for \(6-O\)-methylation prior to deglucosylation by IpeGlu1. Despite co-localization of IpeOMT1 and IpeGlu1 in the cytosol, this may be possible because IpeOMT1 showed a considerably lower \(K_m\) (107 \(\mu\)M) for \(N\)-deacylsoipecoside than did IpeGlu1 (5.8 mM) (15).

The biosynthetic process of glucosidal Ipecac alkaloids having an \(R\)-configuration appears to be more complicated. Considering that ipecoside is accumulated in \(P.\) ipecacuanha regardless of the presence of IpeGlu1 that exhibits the highest catalytic activity toward ipecoside (15), they must be localized in distinct subcellular compartments. Thus, we hypothesized that the \(R\)-diastereomer \(N\)-deacetylipecoside is synthesized and acetylated in the vacuole. This is supported by the fact that non-glucosidal Ipecac alkaloid with an \(R\)-configuration has not been found so far in \(P.\) ipecacuanha. In \(P.\) ipecacuanha, however, \(6-O\)-methylipoce iside and \(7-O\)-methylipoce iside are also accumulated (2). Since IpeOMT2 accepted \(N\)-deacetylipecoside but not ipecoside as substrate, \(6-O\)-methylipoce iside and \(7-O\)-methylipoce iside are presumed to be formed via \(N\)-acytlation of \(6-O\)-methyl-\(N\)-deacetylipecoside and \(7-O\)-methyl-\(N\)-deacetylipecoside, respectively. For this to occur, \(N\)-deacetylipecoside has to be transported into the cytosol to react with IpeOMT2. \(N\)-deacetylipecoside, however, can then react with the cytosolic IpeGlu1, which exhibits a high catalytic activity towards it. Moreover, \(6-O\)-methyl-\(N\)-deacetylipecoside and \(7-O\)-methyl-\(N\)-deacetylipecoside produced need to be transported into the vacuole again based on the above-mentioned hypothesis that \(N\)-acytlation occurs in the vacuole. Discrepancies in subcellular localization between substrate and the corresponding enzyme in natural product biosynthesis have also been reported for DIBOA-Glc metabolizing enzymes in maize, where the substrate DIBOA-Glc was considered to be sequestered in the vacuole, but two enzymes (2-oxoglutarate-dependent dioxygenase and \(O\)-methyltransferase) metabolizing DIBOA-Glc were localized in cytosol (35). As exemplified by anthocyanin transport into the vacuole by a multidrug resistance-associated protein (MRP)-type ATP-binding cassette (ABC) transporter in maize (36, 37), the mechanism of vacuolar transport of plant secondary metabolites is becoming unveiled (38). To reveal the subcellular network of Ipecac alkaloid biosynthetic enzymes, it is essential to identify such transporters, as well as to determine subcellular localization of all biosynthetic enzymes.

Moreover, the cell-specific expression of the biosynthetic genes must be taken into consideration. Immunofluorescence analysis of the morphine biosynthetic enzymes in opium poppy \(P.\) somniferum demonstrated that in capsule and stem, \(3'\)-hydroxy-\(N\)-methylcoclaurine 4\(^{\prime}\)-OMT, reticuline 7-OMT and salutaridinol 7-\(O\)-acetyltransferase were found predominantly in parenchyma cells within the vascular bundle, whereas codeinone reductase is localized to laticifers (39). Accordingly, we can hypothesize that Ipecac alkaloids having the \(S\)- and \(R\)-configuration would be synthesized in distinct types of cells: the former cells need to co-express IpeOMTs and IpeGlu1 in an identical cell to complete the emetine biosynthesis, but the latter might express IpeOMT2 and an enzyme for \(N\)-acytlation but not IpeGlu1 to avoid deglucosylation of \(N\)-deacetylipecoside and its IpeOMT2 products, as well as their acetylated products.

**Functional Differentiation of IpeOMTs in the Evolution of \(P.\) ipecacuanha**—Since IpeOMTs catalyzed \(O\)-methylation reactions of \(6\)- and/or \(7\)-hydroxy groups of the isoquinoline skeleton of Ipecac alkaloids, we expected that each of the three IpeOMTs shows the closest relationship according to its regio-specificity to the known \(6\)- and \(7\)-OMTs for benzylisoquinoline alkaloid biosynthesis. Phylogenetic analysis, however, demonstrated that three IpeOMTs are closely related to each other and the clade is closer to the flavonoid OMTs and 16-hydroxytabersonine OMT than to those benzylisoquinoline alkaloid OMTs (Fig. 2). Similarly, norreticuline 7-OMT showed a closer relationship to norcoclaurine 6-OMT rather than to reticuline 7-OMT in \(P.\) somniferum (19). The authors hypothesized that it would be because 7-OMT had been generated by duplication and the following speciation of 6-OMT locus in the evolutionary process. This hypothesis is supported by the fact that IpeOMTs having distinct
regio-specificity showed the closest relationship to each other. It is most likely that they have evolved in *P. ipecacuanha* after the differentiation from its ancestor through duplications and speciation of an ancestral form of OMT. Since Ipecac alkaloids are produced not only in the genus *Psychotria*, but also in the genus *Alangium* in spite of their different plant families (Rubiaceae and Alangiaceae, respectively), molecular and enzymatic characterizations of Ipecac alkaloid OMTs in *Alangium* should give an important clue to the evolution not only of Ipecac alkaloid OMTs, but also of plant OMTs involved in natural product biosynthesis in general.

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FOOTNOTE
The nucleotide sequences reported in this paper (IpeOMT1-IpeOMT3) has been submitted to the GenBank/EMBL/DDBJ databases with accession numbers AB527082 – AB527084.

ACKNOWLEDGMENTS
We greatly thank Dr. Peter Spiteller (Technische Universität München, Germany) for measuring NMR spectra. We are grateful to Drs. Monica Schmidt and Howard R. Berg (Donald Danforth Plant Science Center) for instrumental and technical support in transient expression experiments, and to Dr. Sona Pandey (Donald Danforth Plant Science Center) for technical instruction in qRT-PCR experiments. We thank Drs. Isabel Ordiz and Nigel Taylor and Brian Kelly (Donald Danforth Plant Science Center) for the gifts of reporter plasmids. We are grateful to Dr. Meinhart H. Zenk (Donald Danforth Plant Science Center) for providing ipecoside, cephaeline, kaempferol, myricetin and benzylisoquinoline alkaloids. We appreciate the gifts of resveratrol and quercetin from Dr. Oliver Yu (Donald Danforth Plant Science Center) and of 7’-O-demethylcephaeline from Dr. Takao Tanahashi (Kobe Pharmaceutical University, Japan). We also thank the Tissue Culture Facility of Donald Danforth Plant Science Center for maintaining the root cultures of P. ipecacuanha. T.N. was supported by a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad.
FIGURE LEGENDS

Figure 1. Schematic representation of the biosynthetic pathway of emetine, cephaeline and related alkaloidal glucosides in P. ipecacuanha. Probable O-methylation steps in Ipecac alkaloid biosynthesis are shown. IpeGlu1 has been identified by ref. 15. The alkaloidal glucosides having an R-configuration, methylalangiside and 6,7-O,O-dimethylisopecoside, both of which have two methoxy groups on the isoquinoline skeleton, have not yet been identified in P. ipecacuanha.

Figure 2. Unrooted phylogenetic tree of the Ipecac alkaloid O-methyltransferases (IpeOMT1, IpeOMT2 and IpeOMT3) with selected members of plant O-methyltransferases. Full-length amino acid sequences were aligned using the ClustalW (version 1.83; http://clustalw.ddbj.nig.ac.jp/top-j.html). The phylogenetic tree was built based on calculation by the neighbor-joining method (40) with bootstrap analysis of 1000 replicates and was visualized with the Treeview (version 1.66). The numbers at each node are bootstrap values per 1000 trials (values over 70% are shown). The scale bar indicates the substitutions per site. The accession numbers for the proteins listed are AAM97497 (C. roseus, flavonoid OMT), ABR20103 (C. roseus, 16-hydroxytabersonine 16-OMT), AAR02419 (C. roseus, flavonoid 4’-OMT), CAQ76879 (V. vinifera, resveratrol OMT), AAB71213 (Prunus armeniaca, OMT), AAM23004 (Rosa hybrid cultivar, orcinol OMT), BAA86059 (Pyrus pyrifolia, OMT), AAL30423 (Ocimum basilicum, chavicol OMT), BAB08005 (C. japonica, 3’-hydroxy-N-methylcoclaurine 4’-OMT), BAB08004 (C. japonica, norcoclaurine 6-OMT), AAQ01699 (P. somniferum, norcoclaurine 6-OMT), ACN88562 (P. somniferum, norreticuline 7-OMT), AAC49708 (Pinus taeda, caffeic acid OMT), AAQ01668 (P. somniferum, reticuline 7-OMT), BAA06192 (C. japonica, scoulerine 9-OMT), AAA33032 (Masembryanthemum crystallinum, myo-inositol OMT), CAA50561 (Nicotiana tabacum, catechol OMT), CAA13175 (Saccharum officinarum, caffeic acid OMT), AAQ01670 (P. somniferum, catechol OMT), AAB46623 (Medicago sativa, caffeic acid OMT), AAD38189 (O. basilicum, caffeic acid OMT), AAA86718 (Zinnia violacea, caffeic acid OMT), AAC01533 (Clarkia breweri, (iso)eugenol OMT), AAB96879 (Arabidopsis thaliana, caffeic acid/5-hydroxyferulic acid OMT), AAA80579 (Chrysosplenium americanum, flavonoid 3’-OMT), AAD29841 (T. tuberosum, norcoclaurine 6-OMT1), AAD29842 (T. tuberosum, norcoclaurine 6-OMT2), AAB48059 (M. sativa, isoliquiritigenin 2’-OMT), BAA13683 (Glycyrrhiza 5-hydroxyechinata, OMT), BAC22084 (C. japonica, columbamine 2-OMT), AAC49856 (Pisum sativum, 6a-hydroxymaackiain OMT), AAC49928 (M. sativa, isoflavone OMT), CAA54616 (Hordeum vulgare, flavonoid 7-OMT), ABY59051 (Zea mays, TRIBOA-glucoside 7-OMT), AAD10485 (Triticum aestivum, OMT), and P47917 (Z. mays, OMT).

Figure 3. SDS-PAGE of the recombinant IpeOMT enzymes expressed in E. coli. Proteins were separated on 12% SDS-PAGE and stained with Coomasie Brilliant Blue G-250. Lane M, molecular size marker; lane C, crude extract from the recombinant E. coli; lane P, purified recombinant enzyme by metal chelation chromatography.

Figure 4. LC-MS/MS analyses of the enzymatic reaction mixture of IpeOMT1-IpeOMT3 with Ipecac alkaloids. Total ion chromatogram (left panel) and the MS/MS fragmentation (right panel(s)) of the reaction product(s) are shown with those of standard compounds. (A) Reaction mixtures of IpeOMT1 and IpeOMT2 with N-deacetylisopecoside, where 6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside are not separated. (B) N-Boc derivatives of the reaction products of N-deacetylisopecoside by IpeOMT1 and IpeOMT2, where 6-O-methyl-N-Boc-deacetylisopecoside and 7-O-methyl-N-Boc-deacetylisopecoside are separated. (C) Reaction mixture of IpeOMT2 with N-deacetylisopecoside, where demethylalangiside, 3-O-demethyl-2-O-methylalangiside and alangiside are lactams non-enzymatically formed from substrate and products. (D) Reaction mixture of IpeOMT1 with 7-O-methyl-N-deacetylisopecoside. (E) Reaction mixture of IpeOMT2 with demethylalangiside. (F) Reaction mixture of IpeOMT2 with 7’-O-demethylcephaeline. (G) Reaction mixture of IpeOMT1 with...
cephaeline. (H) Reaction mixture of IpeOMT2 and IpeOMT3 with redipecamine and 3-O-methylredipecamine, respectively.

Figure 5. Subcellular localization of IpeOMT1-GFP, IpeOMT2-GFP, IpeOMT3-GFP and IpeGlu1-GFP fusion proteins. Onion epidermis was bombarded with DNA-coated gold particles. GFP/YFP fluorescence (left panel) and GFP/YFP-transmission light overlay (right panel) are shown. Non-targeted GFP is the bombardment with empty GFP vector (pBA103). Cytosol-targeted YFP, ER-targeted GFP and vacuole-targeted YFP were also bombarded as controls. Fluorescence signals of IpeOMT-GFPs, IpeGlu1-GFP, non-targeted GFP and cytosol-targeted YFP in the nucleus are attributable to free diffusion of the protein (41).

Figure 6. Time-course analysis of the transcript level of IpeOMT1, IpeOMT2, IpeOMT3 and Ipeglu1 genes by quantitative real-time RT-PCR in P. ipecacuanha root culture. Precultured roots were transferred into new media, and the roots were harvested after each time period from 1 day to 8 weeks. Data are expressed as the mean of triplicate experiments with standard deviation.

Figure 7. Biosynthetic pathway of Ipecac alkaloids having an S-configuration that derive from N-deacetylisoipeicoside. The biosynthetic reactions identified in the present study (IpeOMTs) and IpeGlu1 (15) are shown. Bold arrows indicate emetine biosynthetic pathway. The detailed pathway leading to protoemetine from the aglycon formed by IpeGlu1 has not yet been elucidated (shown by dotted line). Reactions not catalyzed by the IpeOMT enzymes are shown by cross mark.

Figure 8. Biosynthetic pathway of Ipecac alkaloids having an R-configuration that derive from N-deacetylisopecoside. 6,7-O,O-Dimethyl-N-deacetylpeicoside and the lactam methylalangiside not yet detected in Psychotria are shown in gray. Note that methylalangiside has been isolated in Alangium (31, 32). Reactions not catalyzed by the IpeOMT enzymes are shown by cross mark.
| Substrate Tested | Stereochemistry | IpeOMT1 | IpeOMT2 | IpeOMT3 |
|------------------|-----------------|---------|---------|---------|
| **Ipecac alkaloids** | | | | |
| 1 N-Deacetylisopecoside | l(S) | 6-OMT (11) | 6-OMT > 7-OMT (0.14) | - |
| 2 N-Deacetylipecoside | l(R) | - | 6-OMT < 7-OMT | - |
| 3 Ipecoside | l(R) | - | - | - |
| 4 6-O-Methyl-N-deacetylisopecoside | l(S) | - | - | - |
| 5 6-O-Methyl-N-deacetylipecoside | l(R) | - | - | - |
| 6 7-O-Methyl-N-deacetylisopecoside | l(S) | 6-OMT (21) | - | - |
| 7 7-O-Methyl-N-deacetylipecoside | l(R) | - | - | - |
| 8 Demethylisoalangiside | 13a(S) | - | - | - |
| 9 Demethylalangiside | 13a(R) | - | 2-OMT (1.7) | - |
| 10 Isoalangiside | 13a(S) | - | - | - |
| 11 Alangiside | 13a(R) | - | - | - |
| 12 3-O-Demethyl-2-O-methylalangiside | 13a(S) | - | - | - |
| 13 3-O-Demethyl-2-O-methylalangiside | 13a(R) | - | - | - |
| 14 7'-O-Demethylcephaeline | 13a(S) | - | 7'-OMT (51) | - |
| 15 Cephaeline | 11b(S) | 6'-OMT (0.31) | - | - |
| 16 Redipecamine | 13a(S) | - | 2-OMT (2.9) | - |
| 17 2-O-Methylredipecamine | 13a(S) | - | - | - |
| 18 3-O-Methylredipecamine | 13a(S) | - | - | 2-OMT (12) |
| **Benzylisoquinoline alkaloids** | | | | |
| 19 Coclaurine | 1(S) | - | - | 7-OMT (1.9) |
| 20 Isococlaurine | 1(R,S) | 6-OMT (32) | 6-OMT (0.35) | - |
| 21 N-Methylcoclaurine | 1(R,S) | - | - | 7-OMT (0.88) |
| 22 4'-O-Methylcoclaurine | 1(R,S) | - | - | 7-OMT (1.0) |
| 23 Norcoclaurine | 1(R,S) | 6-OMT (15) | 6-OMT (0.60) | - |
| 24 Laudanine | 1(R,S) | - | - | - |
| 25 6-O-Methyltaudanosoline | 1(R,S) | - | - | 7-OMT (0.85) |
| 26 4'-O-Methyltaudanosoline | 1(S) | 6-OMT (0.23) | - | - |
| 27 Nororientaline | 1(R,S) | 4'-OMT (1.0) | - | 7-OMT (2.2) |
| 28 Isoorientaline | 1(R,S) | 6-OMT (3.5) | 6-OMT (0.94) | - |
| 29 Norprotosinomenine | 1(S) | 6-OMT (11) | - | - |
|   | Substrate                      | 1(R) | 6-OMT (nmol·min⁻¹·mg protein⁻¹) | 7-OMT (nmol·min⁻¹·mg protein⁻¹) |
|---|-------------------------------|------|---------------------------------|---------------------------------|
| 30 | Norprotosinomenine            | 1(R) | -                               | -                               |
| 31 | Protosinomenine               | 1(R,S)| 6-OMT (0.72)                    | -                               |
| 32 | Norreticuline                 | 1(S) | -                               | -                               |
| 33 | Norreticuline                 | 1(R) | -                               | -                               |
| 34 | Reticuline                    | 1(S) | -                               | -                               |
| 35 | Reticuline                    | 1(R) | -                               | -                               |
| 36 | Coreximine                    | 13a(S)| -                               | 2- or 11-OMT (0.88)              |
| 37 | Oripavine                     |      | 3-OMT (9.0)                     | -                               |
| 38 | Codeine                       |      | -                               | -                               |
| 39 | Morphine                      |      | -                               | -                               |

Substrates 40-52 (phenolics 40-48, flavonols 49-51 and stilbene 52) are not listed due to absence of enzymatic activity toward all of them. See supplemental Fig. S1 for chemical structures of each substrate. Number in parenthesis is the specific activity (nmol·min⁻¹·mg protein⁻¹) at 0.2 mM substrate. SAM concentration was 1 mM for all assays.

- See supplemental Fig. S1 for corresponding chiral center.
- Activity was calculated from total amount of the two products (6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside), which were not separated under HPLC conditions tested. Their identification was conducted by separating the N-Boc derivatives.
- Activity was not detected.
- Activity was not determined due to non-enzymatic lactamization of substrate and products.
- Synthetic Ipecac alkaloid aglycon mimic.

Activity was calculated from total amount of the two products (6-O-methyl-N-deacetylisopecoside and 7-O-methyl-N-deacetylisopecoside), which were not separated under HPLC conditions tested. Their identification was conducted by separating the N-Boc derivatives.

Activity was not detected.

Activity was not determined due to non-enzymatic lactamization of substrate and products.

Synthetic Ipecac alkaloid aglycon mimic.
**TABLE 2**
Kinetic parameters of the IpeOMT1-IpeOMT3 enzymes.

| Enzyme | Substrate                     | $K_m$ (µM) | $V_{max}$ (nmol·min⁻¹·mg⁻¹) | $k_{cat}$ (s⁻¹, x10⁻³) | $k_{cat}/K_m$ (s⁻¹·M⁻¹) |
|--------|-------------------------------|------------|-----------------------------|------------------------|--------------------------|
| IpeOMT1| $N$-Deacetylisoipecoside $^a$ | 107 ± 4    | 16.6 ± 0.3                  | 11.3 ± 0.2             | 106                      |
|        | $7'$-$O$-Methyl-$N$-deacetylisoipecoside $^a$ | 105 ± 4 | 30.0 ± 0.8                  | 20.4 ± 0.5             | 194                      |
|        | Cephaeline $^a$                | 80 ± 7     | 0.41 ± 0.01                 | 0.28 ± 0.007           | 3.5                      |
|        | SAM $^b$                      | 42 ± 6     | 10.5 ± 0.5                  | 7.1 ± 0.3              | 169                      |
| IpeOMT2| $N$-Deacetylisoipecoside $^a$ | 420 ± 17   | 0.50 ± 0.01                 | 0.34 ± 0.007           | 0.81                     |
|        | $N$-Deacetylipecoside $^a$     | n.d.$^c$  | n.d.$^c$                    | n.d.$^c$               | n.d.$^c$                 |
|        | Demethylalangiside $^a$       | 450 ± 6    | 5.6 ± 0.1                   | 3.8 ± 0.07             | 8.4                      |
|        | Redipecamine $^a$             | 35 ± 1     | 3.4 ± 0.06                  | 2.3 ± 0.04             | 66                       |
|        | $7'$-'O$-Demethylcephaeline $^a$ | 1.0 ± 0.5 | 47.6 ± 2.7                 | 32.6 ± 1.9             | 32600                    |
|        | SAM $^b$                      | 20 ± 1     | 0.38 ± 0.006                | 0.26 ± 0.004           | 13                       |
| IpeOMT3| $3'$-$O$-Methylredipecamine $^a$ | 64 ± 4 | 15.0 ± 0.2                  | 10.5 ± 0.1             | 159                      |
|        | SAM $^d$                      | 34 ± 3     | 12.8 ± 0.4                  | 8.9 ± 0.3              | 262                      |

$^a$ Reaction was performed at 1 mM SAM.

$^b$ Reaction was performed at 0.7 mM $N$-deacetylisoipecoside.

$^c$ Not determined due to non-enzymatic lactamization of substrate and products.

$^d$ Reaction was performed at 0.5 mM 3-$O$-methylredipecamine.
Figure 3 (Nomura and Kutchan)
Figure 4 (Nomura and Kutchan)

A

1. N-Deacetylsiopeicoside / IpeOMT1
   - Retention time: 7.36 min
   - MS/MS: Rt. 7.36 min
   - MS/MS: m/z 376.1

2. N-Deacetylsiopeicoside / IpeOMT2
   - Retention time: 7.37 min
   - MS/MS: Rt. 7.37 min
   - MS/MS: m/z 376.2

3. Standard
   - Retention time: 7.36 min
   - MS/MS: Rt. 7.36 min
   - MS/MS: m/z 376.1

4. 6-O-Methyl-N-deacetylsiopeicoside
   - Retention time: 7.36 min
   - MS/MS: Rt. 7.36 min
   - MS/MS: m/z 376.1

5. Standard
   - Retention time: 7.20 min
   - MS/MS: Rt. 7.20 min
   - MS/MS: m/z 376.1

6. 7-O-Methyl-N-deacetylsiopeicoside
   - Retention time: 7.20 min
   - MS/MS: Rt. 7.20 min
   - MS/MS: m/z 376.1

B

1. N-Deacetylsiopeicoside / IpeOMT1
   (Boc-derivatized product)
   - Retention time: 15.09 min
   - MS/MS: Rt. 15.09 min
   - MS/MS: m/z 376.1

2. N-Deacetylsiopeicoside / IpeOMT2
   (Boc-derivatized products)
   - Retention time: 14.55 min
   - MS/MS: Rt. 14.55 min
   - MS/MS: m/z 376.1

3. Standards
   - Retention time: 14.55 min
   - MS/MS: Rt. 14.55 min
   - MS/MS: m/z 376.1

4. 7-O-Methyl-N-Boc-deacetylsiopeicoside
   - Retention time: 15.08 min
   - MS/MS: Rt. 15.08 min
   - MS/MS: m/z 376.1

5. 6,7-O-Methyl-N-Boc-deacetylsiopeicoside
   - Retention time: 15.14 min
   - MS/MS: Rt. 15.14 min
   - MS/MS: m/z 376.1
Figure 4 (Nomura and Kutchan) (continued)
Figure 4 (Nomura and Kutchan) (continued)
Figure 5 (Nomura and Kutchan)

|                         | Fluorescence | Fluorescence + transmission light |
|-------------------------|--------------|-----------------------------------|
| IpeOMT1-GFP             | ![Image](image1) | ![Image](image2)                  |
| IpeOMT2-GFP             | ![Image](image3) | ![Image](image4)                  |
| IpeOMT3-GFP             | ![Image](image5) | ![Image](image6)                  |
| IpeGlu1-GFP             | ![Image](image7) | ![Image](image8)                  |
| Non-targeted GFP        | ![Image](image9) | ![Image](image10)                 |
| Cytosol-targeted YFP    | ![Image](image11) | ![Image](image12)                 |
| ER-targeted GFP         | ![Image](image13) | ![Image](image14)                 |
| Vacuole-targeted YFP    | ![Image](image15) | ![Image](image16)                 |
Figure 6 (Nomura and Kutchan)

Molecules (x10^3) / pg of cDNA

- ipeglu1
- ipeOMT1
- ipeOMT2
- ipeOMT3

1 day | 3 days | 1 week | 2 weeks | 4 weeks | 6 weeks | 8 weeks
Figure 8 (Nomura and Kutchan)

6-O-Methyl-N-deacetylpecoside

IpeOMT2

6,7-O-Dimethyl-N-deacetylpecoside

N-Deacetylpecoside

IpeOMT2

7-O-Methyl-N-deacetylpecoside

Spontaneous

Alangiside

Methylalangiside

Demethylalangiside

IpeOMT2

3-O-Demethyl-2-O-methylalangiside

Lactams
Three new O-methyltransferases are sufficient for all O-methylation reactions of ipecac alkaloid biosynthesis in root culture of *Psychotria ipecacuanha*
Taiji Nomura and Toni M. Kutchan

*J. Biol. Chem.* published online January 8, 2010

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