Isolation and characterization of novel *O*-methyltransferase involved in benzylisoquinoline alkaloids biosynthesis in *Eschscholzia californica*

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Contents

Abstract

California poppy (*Eschscholzia californica*), a member of the Papaveraceae family, produces many kinds of pharmacologically active benzylisoquinoline alkaloids (BIAs), such as chelerythrine, sanguinarine, macarpine, and key intermediate reticuline. Among those biological active metabolites, sanguinarine biosynthesis has been well elucidated at the molecular level, whereas several enzyme-encoding genes in the biosynthesis of chelerythrine and macarpine were only partially characterized. In this research, I isolated and characterized a novel *O*-methyltransferase (OMT) involved in the biosynthesis of BIA, especially chelerythrine.

In Chapter I, I searched new OMT candidates. Using cDNA database of NCBI and PhytoMetaSyn of *E. californica*, OMT candidates were searched based on the conserved OMT domain. Sixty eight new OMT-like sequences were found and then grouped into 22 sequences based on their sequence similarity. Furthermore, after evaluation of their expression in cell lines with different chelerythrine/macarpine profile (S-38 and A5-1 cell lines), three OMTs candidates (G2, G3, and G11OMT) were selected. A phylogenetic tree with several known OMTs showed that those three OMTs were in different clades and might have distinct functions in BIA biosynthesis pathway.

In Chapter II, recombinant protein of G3OMT was produced in *E.coli* cells and its enzymological activity to methylate simple benzylisoquinoline alkaloids (reticuline and norreticuline) and a protoberberine (scoulerine) was determined. G3OMT methylated reticuline or norreticuline alkaloids at the 7- and 3'- positions and methylated scoulerine at 2 and 9 positions. Biosynthetic role of G3OMT was further characterized using transgenic *Pichia* cells expressing G3OMT and other biosynthetic enzyme-encoding genes in BIA biosynthesis suggested that G3OMT would have function as scoulerine-9-*O*-methyltransferase in the chelerythrine biosynthesis. Biotechnological potentials of G3OMT were also discussed.

Abbreviations

4’OMT	3’-hydroxy-*N*-methylcoclaurine 4’-*O*-methyltransferase
6OMT	norcoclaurine 6-*O*-methyltransferase
7OMT	reticuline 7-*O*-methyltransferase
AdoMet	*S*-adenosyl-L-methionine
BBE	berberine bridge enzyme
BIA	benzylisoquinoline alkaloid
BME	β-mercaptoethanol
BMMY	buffered methanol-complex
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHES	*N*-cyclohexyl-2-aminoethanesulfonic acid
Cj	*Coptis japonica*
CNMT	coclaurine *N*-methyltransferase
CoOMT	columbamine *O*-methyltransferase
CYP719A2	stylopine synthase
CYP719A3	stylopine/canadine synthase
CYP719A5	cheilanthifoline synthase
DBOX	dihydrobenzophenanthridine oxidase
Ec	*Eschscholzia californica*
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
EST	expressed sequence tag
HEPES	2-[4-(hydroxyethyl)-1-piperazinyl ethanesulfonic acid
HPLC	high performance liquid chromatography
IAA	iodoacetamide
id	inner diameter
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo dalton
LB	Luria Bertani

LC/MS	liquid chromatography-mass spectrometry
MSH	*N*-methylstylopine 14-hydroxylase
ND	not detected
NLS	norlaudanosoline
OD	optical density
ODS	octadecylsilyl
OMT	*O*-methyltransferase
P6H	protopine 6-hydroxylase
PCR	polymerase chain reaction
Ps	*Papaver somniferum*
RT-PCR	reverse transcription-PCR
SAH	*S*-adenosyl-L-homocysteine
SD	standard deviation
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SIM	single ion monitoring
SMT	scoulerine 9-*O*-methyltransferase of *Coptis japonica*
SOMT	scoulerine 9-*O*-methyltransferase of *Papaver somniferum*
SR	sanguinarine reductase
TAPS	*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TIC	total ion chromatograph
THBO	tetrahydroprotoberberine oxidase
TNMT	tetrahydroberberine *N*-methyltransferase
Tricine	*N*-[tris(hydroxymethyl)methyl]glycine
YPD	yeast extract, peptone, dextrose

General Introduction

Natural products from higher plants can be devided into several major groups: terpenoids, phenolic compounds, alkaloids, and cyanogenic glucosides and glucosinolates, which have long been used as dyes, polymers, glues, oils, flavors, and drugs (Kutchan et al., 2015). Alkaloids, a nitrogen-containing compounds, which are usually in a heterocyclic ring, are important natural products that have strong biological activities. Approximately 20% of plant species contain alkaloids and over 12,000 kind of alkaloids have been characterized (De Luca and St. Pierre, 2000). So far, intensive characterization of these bioactive compounds have been conducted to determine their chemical structures, biological activities, biosynthetic pathways, and biosynthetic enzymes and genes (Facchini and De Luca, 2008; Sato, 2013).

One of the useful alkaloids is benzylisoquinoline alkaloids (BIAs). They are produced in many plant families such as Ranunculaceae, Papaveraceae, Berberidaceae, and Menispermaceae (Facchini, 2001; Facchini and De Luca, 2008; Ziegler and Facchini, 2008). BIAs have many diverse chemical structures and pharmacological effects (Ziegler and Facchini, 2008), such as the antimicrobial berberine (a protoberberine) in *Coptis japonica*, the narcotic analgesics morphine and codeine (morphinans) in *Papaver somniferum* and the antimicrobial sanguinarine (a benzophenanthridine) in *Eschscholzia californica*. To characterize their biosynthesis pathways, the enzymes, and the enzyme-coding genes of protoberberine, benzophenanthridine and morphinan pathways have been studied extensively and characterized at molecular level (Hagel and Facchini, 2013; Sato and Kumagai, 2013).

From the ancient time, natural products have been used as main source of medicines and even last 25 years natural products are still one of main sources of drug developments although many molecules have been chemically synthesized (Newman and Cragg, 2007; Leonard et al., 2009). However, limited amounts of plant derived chemicals in nature, lack of geographic access and cultivation, seasonal limitation of production and inefficiency of processing of some bioactive compounds, such as separation and purification lead efforts to find an alternative source and way to produce bioactive compounds (Leonard et al., 2009; Diamond and Desgagne-Penix, 2016). An alternative production system, such as total or semi chemical synthesis, has been developed but has limitation due to the complex structures of natural products (Graening and Schmalz, 2004; Rinner and Hudlicky, 2012). More biological way to produce complex structured natural chemicals using plant breeding, plant cell cultures, and metabolic engineering have been investigated (Sato et al., 2001; Sato et al., 2007; Sato, 2013). Whereas the cultivation of medicinal plants, which produce plant-derived specialized metabolites have been well established and some breedings have been developed, the cultivation of plants are environmental sensitive and production are often fluctuated year by year. Therefore, production in plant cell cultures has been developed. However, productivities of desired metabolites in cell cultures were often low and cultivation was costly because of relatively high cost of long sterile cultivation and scale up (Cho, et al., 2008; Sato, 2013; Verma, et al., 2014). Synthetic biology in microbe for metabolite production may serve as alternative approach to chemical synthesis, plant cell culture or plant biomass extraction. This method use a reconstruction of plant biosynthetic pathways in heterologous host systems and also open possibility to construct a novel biosynthetic pathway that not present naturally in plant. For example,

(*S*)-reticuline was produced in *E. coli* cells that heterogously expressed several enzymes in BIA biosynthesis, such as *Coptis* 6OMT, CNMT, and 4'OMT (Minami et al., 2008). Nakagawa et al. (2016) reported production of thebaine, an opiate alkaloid, from glucose in *E. coli* cells. The reconsctruction of BIAs biosynthesis in yeast were also reported (Hawkins and Smolke, 2008; Fossati et al., 2014; Hori et al., 2016).

Because of its well-characterized biosynthetic pathways, enzymes, and enzyme-coding genes, BIA biosynthesis pathway is good model of metabolite engineering and synthetic biology. Examinations of biosynthetic pathway reconstruction using a recombinant system had been performed to characterize the biosynthetic enzymes. Recent studies (Hori et al., 2016) described that stylopine was synthesized from reticuline using berberine bridge enzyme (BBE, Dittrich and Kutchan, 1991), cheilanthifoline synthase (CYP719A5, Ikezawa et al., 2009), and stylopine synthase (CYP719A2/3, Ikezawa et al., 2007) by the reactions of these enzymes in *Pichia* cells.

California poppy (*Eschscholzia californica*), a member of the Papaveraceae family, produces many BIAs, such as protopine, sanguinarine, macarpine, and chelerythrine, and is a good model to study BIA biosynthesis. Most of the biosynthetic enzymes of those alkaloids have been characterized at the molecular level, especially sanguinarine (a benzophenanthridine alkaloid) production from reticuline. Whereas antimicrobial activity of sanguinarine is well reported, sanguinarine is also reported as a promising anticancer agent (Ahmad et al., 2000; Slaninova et al., 2001) and a potential chemical to reduce lipid accumulation in *C. elegans* (Chow and Sato, 2013). Sanguinarine analog, chelerythrine, is also well-known as a protein kinase inhibitor and has antitumor activity (Chmura et al., 2000). Induction of apoptosis in several cancer cell lines was also reported for chelerythrine (Basu et al., 2013).

BIA biosynthesis in California poppy is derived from tyrosine (amino acid) and involving several enzymes and sequential enzyme reactions, such as decarboxylation, hydroxylation, methylation, berberine ring formation, and reduction. BIA biosynthesis begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde yielding (*S*)-norcoclaurine, a simple BIA, which is converted to reticuline, an important intermediate in BIA biosynthesis. Then, scoulerine, the first closed-ring form in BIA biosynthesis, is produced from reticuline by berberine bridge enzyme (Dittrich and Kutchan, 1991; Liscombe and Facchini, 2007) (Figure 0-1). Then, via several sequential reactions by enzymes such as cheilanthifoline synthase (CYP719A5, Ikezawa et al., 2009), stylopine synthase (CYP719A2/A3, Ikezawa et al., 2007), tetrahydroprotoberberine *N*-methyltransferase (TNMT, Liscombe and Facchini, 2007), *N*-methylstylopine 14-hydroxylase (MSH, Beaudoin and Facchini, 2013), protopine 6-hydroxylase (P6H, Takemura et al., 2013), scoulerine is converted to dihydosanguinarine, a benzophenanthridine alkaloid. Dihydrosanguinarine is further converted to sanguinarine by dihydrobenzophenanthridine oxidase (DBOX, Hagel et al., 2012).

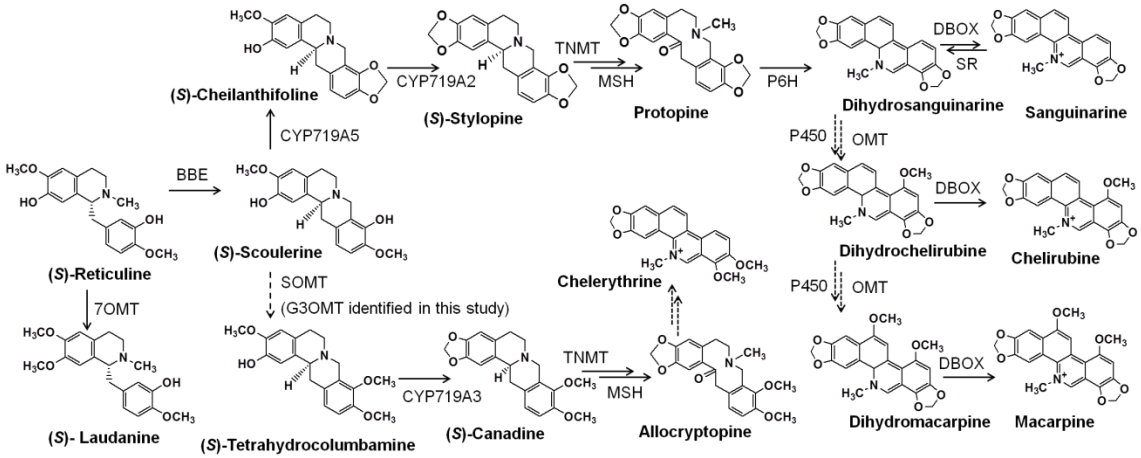


Figure 0-1. Benzylisoquinoline alkaloid biosynthesis in California poppy (partial biosynthetic pathway shown starting from reticuline)
The dotted line indicates that the enzyme-coding genes have not been identified.
Abbreviations: BBE, berberine bridge enzyme; CYP719A5, cheilanthifoline synthase; CYP719A2, stylopine synthase, CYP719A3, stylopine/canadine synthase; TNMT, tetrahydroberberine *N*-methyltransferase; MSH, *N*-methylstylopine 14-hydroxylase; P6H, protopine 6-hydroxylase; SOMT, scoulerine *O*-methyltransferase; DBOX, dihydrobenzophenanthridine oxidase; and SR, sanguinarine reductase

California poppy produces more complex benzophenanthridine alkaloids such as chelerythrine, chelirubine, and macarpine, as shown in Figure 0-1. The biosynthetic enzymes of those alkaloids were partly characterized at the molecular level. Thus, I tried to investigate enzyme-coding genes involved in this partly characterized pathway, especially *O*-methyltransferase (OMT) genes. *O*-methyltransferases methylate an oxygen atom, or transfer methyl group to hydroxyl group, an acceptor molecule, and yield variety of secondary metabolites (Cui et al., 2011). The *O*-methylation of hydroxylated small molecules is crucial for product diversification via branched pathway using same or similar substrates (Zubieta et al., 2001; Morishige et al., 2010; Dang and Facchini, 2012).

Many kinds of OMTs involved in BIAs biosynthesis have been characterized, such as norcoclaurine 6-*O*-methyltransferase (6OMT) (Sato et al, 1994; Morishige et al, 2000), 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT) (Morishige et

al, 2000), columbamine *O*-methyltransferase (CoOMT; Morishige et al., 2002), reticuline 7-*O*-methyltransferase (7OMT in California poppy; Fujii et al., 2007, and *P. somniferum*; Ounaroon et al., 2003), and scoulerine 9-*O*-methyltransferase (*Coptis japonica* SMT; Takeshita et al., 1995, and *P. somniferum* SOMT; Dang and Facchini, 2012). OMTs usually transfer methyl group from *S*-adenosyl-methionine (AdoMet) to substrates, and have a similar sequence structure for conserved AdoMet binding domain (Ziegler and Facchini, 2008; Morishige et al., 2010).

To search biosynthetic OMTs in uncharacterized pathways, such as biosynthesis of chelerythrine and macarpine, I used the conserved domain of known OMTs in California poppy (6OMT, 4'OMT, and 7OMT) and *Coptis japonica* (6OMT, 4'OMT, CoOMT, and SMT) as a query to search candidate genes in the databases of NCBI (www.ncbi.nlm.nih.gov) and the PhytoMetSyn (www.phytometasyn.ca), and found 68 OMT-like sequences. According to their sequence similarities, those sequences were grouped into 22 groups. Their gene expressions were examined by a quantitative RT-PCR in two California poppy cell lines, which had different alkaloid profiles, and three OMT groups with distinct gene expression in the tested two cell lines were selected for further analysis (Chapter I). In Chapter II, the full-length cDNAs of the three OMT candidate genes (G2, G3, G11) were isolated and expressed in *E.coli* using an expression vector to characterize their enzymological properties. Among three OMT genes, only G3OMT showed successful expression and showed unique OMT activity against several alkaloid substrates. That is, G3OMT methylated reticuline and norreticuline at 7 and 3' positions and also produced dual-methylated laudanosine and norlaudanosine, respectively. G3OMT also methylated scoulerine at 2 and 9 positions and produced tetrahydropalmatine as dual-methylated product. Because G3OMT would

be involved in broad alkaloid metabolism, I investigated its role in BIA biosynthesis by co-incubation of G3OMT with several other enzymes in BIA biosynthesis, which were expressed in *Pichia* cells. The co-incubation result suggests that G3OMT function as scoulerine 9-OMT in the biosynthesis of chelerythrine. G3OMT also showed biocatalyst activity to produce new alkaloid products. Based on my investigation, I discuss the characterization of the novel OMT involved in the uncharacterized BIA biosynthesis and its potential for biotechnological application.

Chapter I

Screening of *O*-methyltransferase (OMT) candidate genes in benzylisoquinoline alkaloid (BIA) biosynthesis in California poppy

Introduction

Recently, many plant transcriptome informations have been determined and saved in databases. Especially, several medicinal plant data are stored in NCBI (http://www.ncbi.nlm.nih.gov) and PhytoMetaSyn (www.phytometasyn.ca). The avaibility of transcriptome data for several plant producing BIAs fasilitated a comparative analysis to characterize ortholog enzymes (Xiao et al., 2013). California poppy, a native American's folk medicinal plant, was also sequenced so far. Then, I used these datasets for initial screening to find uncharacterized genes using a known nucleotide sequences of biosynthetic enzymes.

As described in General Introduction, *O*-methyltransferases (OMTs), enzymes responsible for directing *O*-methylation, are key enzymes in the biosynthesis of specialized metabolism and have conserved AdoMet binding domain. Using conserved AdoMet binding domain, OMT candidate genes were searched and 68 genes were listed. These candidates were further grouped into 22 groups based on its similarity/identity.

Whereas sequence informations showed diversification of candidates OMTs, they did not provide the functional information. Therefore, I characterized the gene expression of OMT-like sequences in California poppy cell lines, which have different macarpine/chelerythrine profiles. Then, 3 candidate genes, which have high expression in high macarpine producing cell line, were chosen for further characterization.

Materials and Methods

Plant material

Culture cells of California poppy (*Eschscholzia californica*) with different alkaloid profiles, i.e. A5-1 cell line, a high macarpine producing cell line with over-expressing the rate-limiting *EcCYP719A5* gene (Takemura et al., 2010b), and S-38 cell line, a low macarpine producing but high 10-hydroxychelerythrine producing cell line with over-expressing the *CjSMT* gene (Takemura et al., 2010a), were sub-cultured every three weeks in Linsmaier-Skoog medium containing 10 μM naphthalene acetic acid and 1 μM benzyladenine with 3% sucrose in the dark. Alkaloids were extracted from two-day old culture cells with methanol containing 0.01N HCl and analyzed with LC-MS 2020 (Shimadzu) using the followong system: a TSKgel ODS-80 T_M column (4.6 mm i.d. x 250 mm, 5 μm, TOSOH, Japan), gradient elution with solvent A (1% acetic acid) and solvent B (acetonitrile containing 1% acetic acid) with composition 40% solvent B (0-15 min), 80% solvent B (18-50 min), 40% solvent B (55-60 min), and flow rate 0.5 mL/min at 40^{o}C. The metabolites was monitored by both mass ion signal from 50 to 400 and UV spectrum at 190-600 nm measured by a photodiode array detector.

Total RNA of two-day-old culture cells were prepared using RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and cDNAs were prepared using a PrimeScript RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan) based on the manufacturer's instruction.

Screening of OMT candidate genes and isolation of full-length cDNA

OMT candidate genes were screened from the cDNA databases of NCBI (http://www.ncbi.nlm.nih.gov) and PhytoMetaSyn (www.phytometasyn.ca) using known OMT sequences involved in BIA biosynthesis in California poppy [3'-hydroxy-

N-methylcoclaurine-4'-O-methyltransferase (4'OMT, GenBank AB745041.1), norcoclaurine 6-*O*-methyltransferase (6OMT, GenBank AB745042.1), reticuline 7-*O*-methyltransferase (7OMT, GenBank AB232153.1)] and *Coptis japonica* [3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT, GenBank D29812.1), norcoclaurine 6-*O*-methyltransferase (6OMT, GenBank D29811.1), scoulerine 9-*O*-methyltransferase (SMT, GenBank D29809.1), columbamine *O*-methyltransferase (CoOMT, GenBank AB073908.1)]. The candidate OMTs sequences were searched by BLASTn.

From BLASTn search, 118 of new OMT-like sequences were found. Analysis of these 118 sequences showed that only 68 sequences contained conserved motif of AdoMet binding, whereas 50 sequences were not. Thus, these 68 unique and uncharacterized OMT-like sequences were further characterized based on their sequence identity.

Analysis of 22 group candidate genes expression using quantitative RT-PCR

The expressions of 22 candidate genes were examined in S-38 and A5-1 cell lines using sets of primers for 22 candidate genes (Table 1-1). The quantitative RT-PCR reactions were performed using iQ™ SBYR® Green Supermix (Bio-Rad) to produce approximately 80-170 bp fragments and 40 cycles of the following program: 10 sec at 95°C, 10 sec at 58.5°C, and 20 sec at 72°C using cDNAs as described above. Actin was used as the housekeeping gene to normalize the expression of the OMT candidate genes.

Table 1-1. Primer sequences which were designed by primer3 plus software for RT-PCR to measure the gene expression of 22 OMT groups in S-38 and A5-1 cell lines.

OMTs candidates	**Forward primer (5' to 3')**	**Reverse primer (5'to 3')**
Group 1	GTGCCGTGGTACTCGAATG	TGTTCAATGGCTCCTCTGC
Group 2	GGATCTGTTCCATGGTACTCAA	AGGTCCTTATGGAAAGCTGGTA
Group 3	CCTCGTAGTGGATGCTATATTGG	GCCTCTTATATGCGGGTATTTCT
Group 4	AAGTCGAAATTGGTTCCCTTG	GCCACCATAACCATGAAGAAA
Group 5	TAAACCCGATGCAGATGTGA	AACGGTGCAAGGTTGAAATC
Group 6	AGCGGATCTTTGTCGTTCATA	GCCATGGATGATTCTGAGCTA
Group 7	TATAGATTGGGTTGCCGGAATA	AGAAAGGACTGAAGTCGAGTGG
Group 8	GGAATAATCGAGCTTTCCGTTA	CACCTCCAATCAAGACACACAT
Group 9	CAGTCTCGAACGCTGTTGAA	GTTCTCCTTGGCACCTTTTG
Group 10	TTTATTGCACCTTCGTCGTTC	GGTGGTGACATGTTTGAATCC
Group 11	CTGTGGCTCATGTCATTTGTG	AATGGATTCGAACATGTCACC
Group 12	CTCTAGTCCTCCGTTGTGCAG	TTGAGATTGGGAGCTTTGATG
Group 13	GTTTCACCTTCCATTCCAACA	GAAGGATTGGGTGAAGGAAGTA
Group 14	GGCCACGAGTCTCTAATTGAA	CAAACACCACCATAGTCTCATCA
Group 15	AATGGTGTCGGAGAATCGTC	TTGAAGGATTGGGTTCTTGG
Group 16	GGTCGACTTAACTTTGGTACGC	ACCCACTACCCACATAACCATC
Group 17	ATGACATCGGTATCGGTAGAAGA	GACACTAGTAGACGTCGGTGGAG
Group 18	ACAGACGGCTAAGGATAAACCTC	GATGGTGCTGACTGTACTTGATG
Group 19	GACGGATACAAGCATATCACCA	TAGGCTCTGCTGCTAAGGCTAT
Group 20	TCCAACACCACCAAGATTCA	ACCGTTGGCTTTAACGAATG
Group 21	AGAGAGGGAGTCGAAACATCC	GGAATCCAGCATCATCGAATA
Group 22	TGAAGCTGTAGCTATTGGACCTC	AAAGCTTCCATATCAGGAAGGAG

Phylogeny tree of the OMT candidate genes

Based on our unpublished draft genome data of California poppy, sets of primers with *BamHI* site (GGATCC) were designed to isolate the full-length cDNAs of those genes (Table 1-2). G2OMT is expected to encode 365 amino acids, whereas G3 and G11OMT encode 362 and 352 amino acids, respectively.

Table 1-2. Primer sequences used to isolate full lenght cDNAs of G2, G3, and G11 OMT genes

OMTs candidates	**Forward primer (5'to 3')**	**Reverse primer (5'to 3')**
Group 2	GGATCCATGGGTTCAACAGA AAACCA	GGATCCTTAGTTCTTAGTGA ACTCCATAACAA
Group 3	GGATCCATGGAGAAGGGAA AATTAGAGG	GGATCCTTAAATATCAGGGT AAGCCTCAA
Group 11	GGATCCATGAATTCTCAAAC AGAGATGATG	GGATCCTTAAGGAAAAGCTT CAATAATAGAG

PCR products were subcloned into a pGEM-T easy vector (Promega), and their sequences were determined by Fasmac Co. Ltd (Japan). The nucleotide sequences were submitted to DDBJ/GenBank/EMBL with accession number LC171866, LC171865, and LC171864, for G2, G3, and G11 OMT, respectively.

Phylogenetic tree was built using full-length amino acid sequences with unrooted phylogenetic relationship, neighbor-joining statistical method, Poisson model, and 1000 bootstrap replications. For the analysis, following known OMT sequences were used in comparison with G2, G3, and G11: PsSOMT1, scoulerine 9OMT-1 (*Papaver somniferum*, AFB74611.1); PsSOMT2, scoulerine 9OMT-2 (*P. somniferum*, AFB74612.1); PsSOMT3, scoulerine 9OMT-3 (*P. somniferum*, AFB74613.1); Cj4'OMT, 4'OMT (*Coptis japonica*, BAB08005.1); Cj6OMT, norcoclaurine 6OMT (*C. japonica*, BAB08004.1); CjSMT, scoulerine 9OMT (*C. japonica*, BAA06192.1);

CjCoOMT, tetrahydrocolumbamine 2OMT (*C. japonica*, Q8H9A8.1); Ec4'OMT, 4'OMT (*Eschscholzia californica*, BAM37633.1); Ec6OMT, norcoclaurine 6OMT (*E. californica*, BAM37634.1); Ec7OMT, reticuline 7OMT (*E. californica*, BAE79723.1); Ps7OMT, reticuline 7OMT (*P. somniferum*, AAQ01668.1); TtCaOMT, catechol OMT (*Thalictrum tuberosum*, AAD29843.1); Tf6OMT, norcoclaurine 6-OMT (*Thalictrum flavum*, AAU20765.1); PsCaOMT, catechol OMT (*P. somniferum*, AAQ01670.1); Tf4'OMT, 3'-hydroxy-*N*-methylcoclaurine 4'OMT (*T. flavum*, AAU20768.1); VvReOMT, resveratrol OMT (*Vitis vinifera*, CAQ76879.1); TfSMT, scoulerine 9OMT (*T. flavum*, AAU20770.1); CbCafOMT, caffeate OMT (*Cardamine breweri*, O23760.1); AmCafOMT, caffeate OMT (*Ammi majus*, AAR24095.1); CbEuOMT, isoeugenol OMT (*C. breweri*, AAC01533.1); VvCaOMT, caffeic acid OMT (*V. vinifera*, AAF44672.1); MsCaOMT, caffeic acid 3OMT (*Medicago sativa*, AAB46623.1); IpeOMT1, IpeOMT-1 (*Carapichea ipecacuanha*, BAJ05383.1); Cr16OMT, 16-hydroxytabersonin OMT (*Catharanthus roseus*, ABR20103.1); ObCafOMT, caffeate OMT (*Ocimum basilicum*, AAD38189.1); NtCaOMT, catechol OMT (*Nicotiana tabacum*, CAA50561.1); HvFl7OMT, flavonoid 7-OMT (*Hordeum vulgare*, CAA54616.1); McInOMT, inositol OMT (*Mesembryanthemum crystallicum*, AAB05891.1); RcOrOMT, orcinol OMT-1 (*Rosa chinensis*, CAH05077.1); MsLiOMT, isoliquiritigenin 2'-OMT (*M. sativa*, AAB48059.1); AtQu3'OMT, quercetine 3'-OMT (*Arabidopsis thaliana*, Q9FK25.1); ObEuOMT, eugenol OMT (*O. basilicum*, AAL30424.1), and PsFl4'OMT, isoflavone 4'-OMT (*Pisum sativum*, O24305.1).

Results

Alkaloid profiles of S-38 and A5-1 cell lines

S-38 cells showed high 10-hydroxychelerythrine but low macarpine alkaloid accumulation, whereas A5-1 showed high macarpine alkaloid accumulation (Figure 1-1).

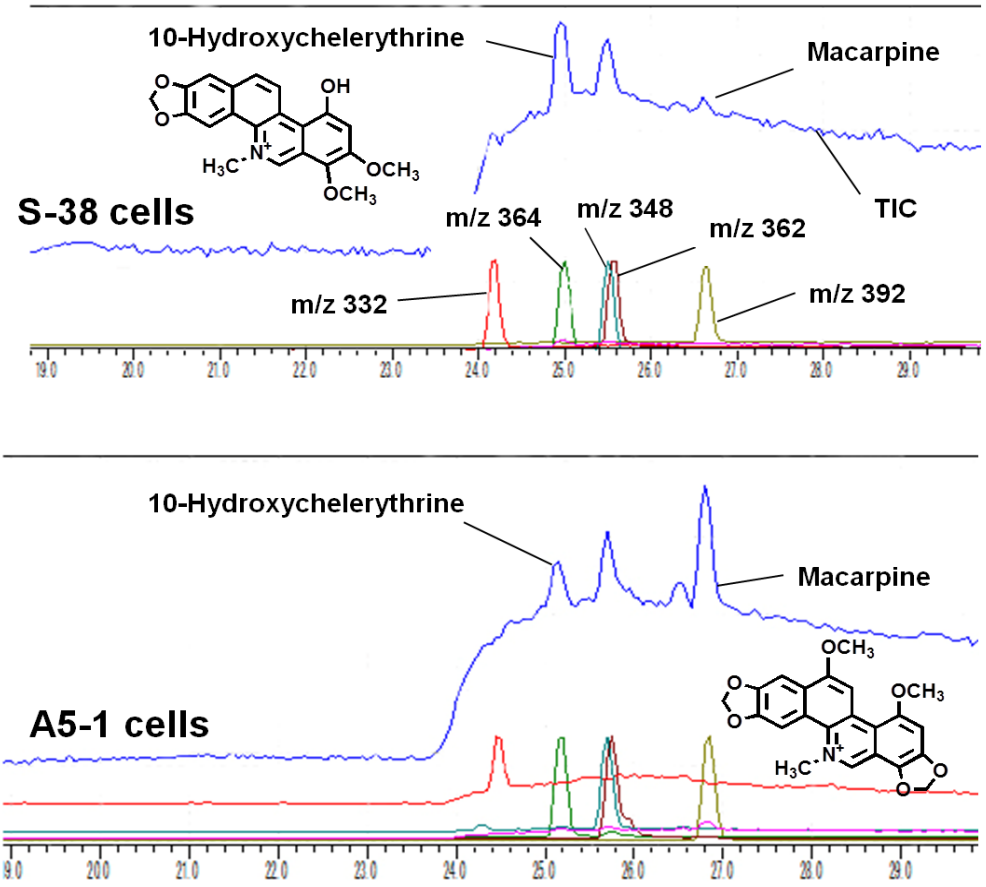


Figure 1-1. Alkaloid profiles of S-38 and A5-1 cell lines determined by LC-MS 2020. Dark blue line indicates TIC (Total Ion Chromatograph), red is SIM (Single Ion Monitoring) of m/z 332 (sanguinarine), green is SIM of m/z 364 (10-hydroxychelerythrine), light blue is SIM of m/z 348 (chelerythrine), brown is SIM of m/z 362 (chelirubine), and gold is SIM of m/z 392 (macarpine).

Screening of OMT candidate genes

As described in Material and Methods in this chapter, sixty eight of unique and uncharacterized OMT-like sequences were found in transcriptome data in NCBI and PhytoMetaSyn. Because the amino acids of 68 sequences were not full-length, phylogeny analysis only used the sequences between conserved motif I to III (approximately 110 amino acids). Phylogeny tree analysis showed 68 sequences were grouped into 22 representative candidate groups (Figure 1-2). Among 22 group genes, the longest 22 representative gene candidates were selected for further expression analysis.

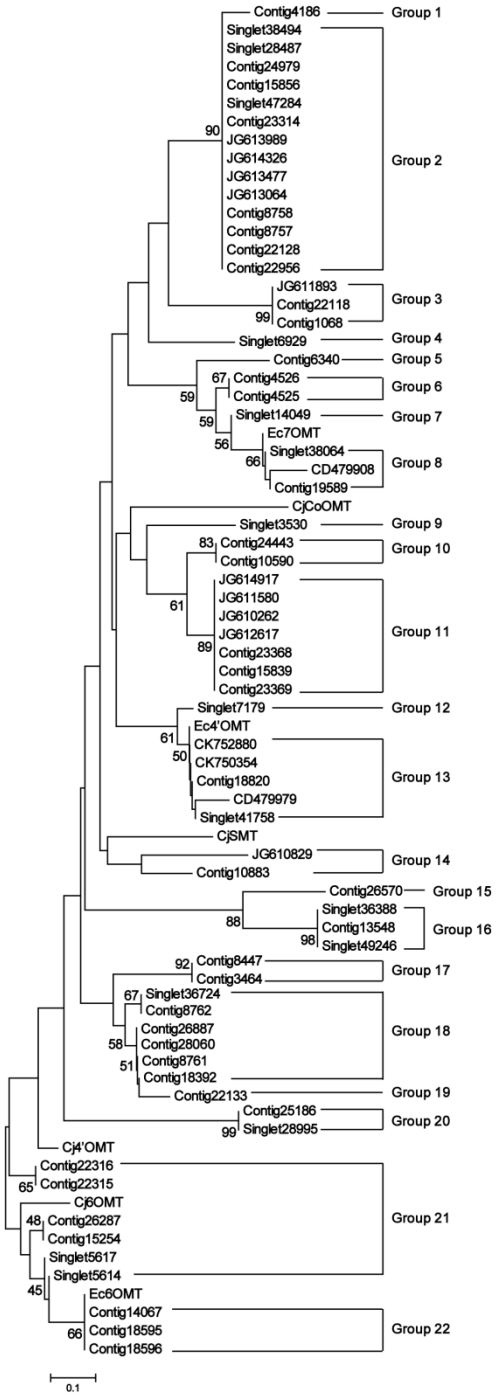


Figure 1-2. Phylogenetic tree analysis of 68 amino acid sequences of OMT-like sequences which was analyzed based on the sequence identity of the conserved motifs (motif I-III) of the OMTs by the neighbor-joining statistical method, Poisson model, and 1000 of bootstrap replication number. The branch length is proportional to the estimated divergence distance of each amino acid. The scale bar (0.1) means a 10% change. The numbers of replicate tree percentage associated with taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Examination of gene expression level using quantitative RT-PCR

Using each gene specific primer sets (Table 1-1), expression of 22 candidate genes was determined by quantitative RT-PCR in high macarpine producing A5-1 cells and low macarpine producing S-38 cells. Actin gene was used to normalize the expression.

As shown in Figure 1-3, groups 1, 4, 5, 7, 9, 16, 18, and 19 did not show any amplification products, whereas Group 6, 8, 10, 12-15, 17, 20, 21, and 22 showed similar expression in both A5-1 and S-38 cell lines. On the other hand, Group 2, 3, and 11 showed high expression in A5-1 cell line and low expression in S-38 cell line (more than 5-fold of difference). Thus, three candidate genes (Group 2, 3, and 11 which called as G2, G3, and G11, respectively) were selected to characterize their role in macarpine biosynthesis.

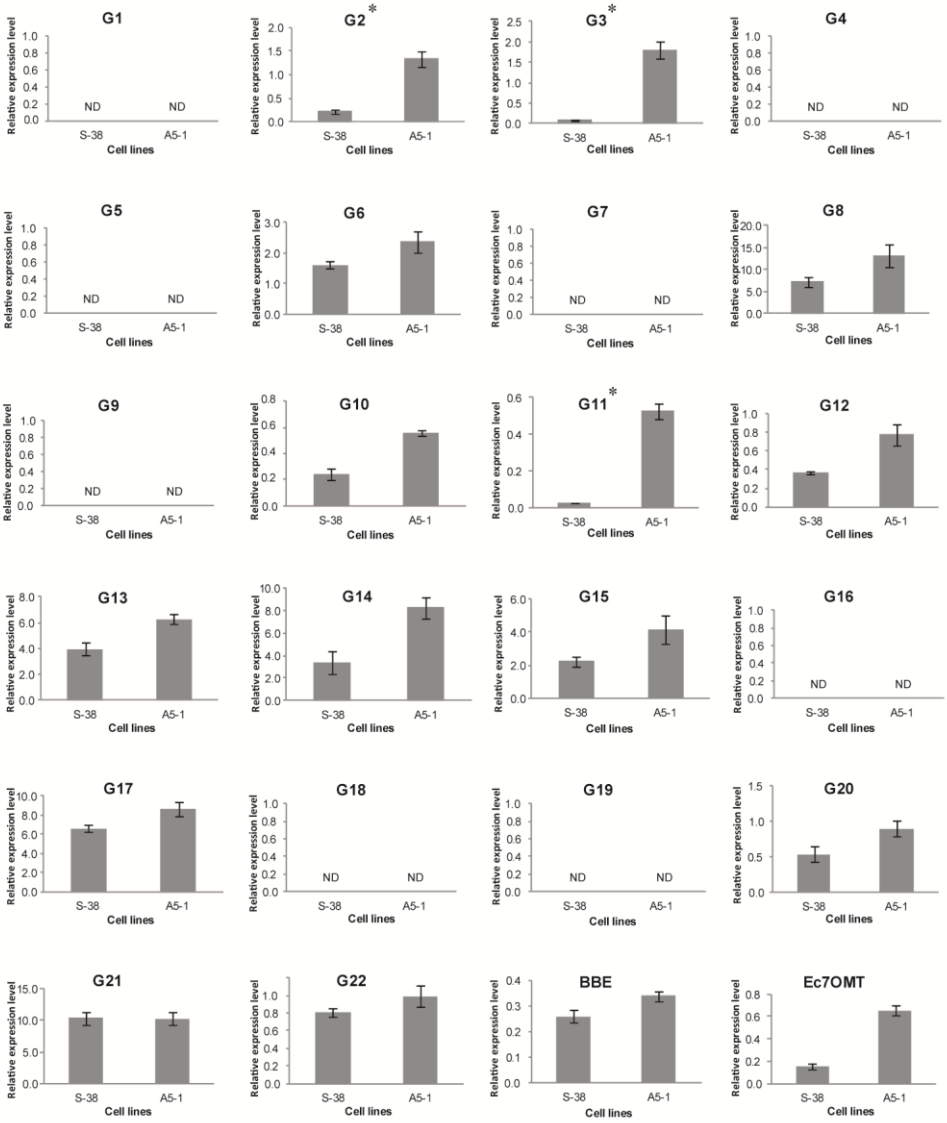


Figure 1-3. Quantitative RT-PCR of the OMT candidate genes expression in S-38 (low macarpine cells) and A5-1 (high macarpine cells). Each value represents the mean ± SD of three replicates. ND: not detected. Values in each panel indicates the relative expression of each gene in A5-1 in comparison with that in S-38. Asterisk indicates more than 5-fold of different of gene expression in two cell lines.

Phylogenetic tree of the OMT candidate genes

G2OMT had 76% and 73% identity to catechol OMT from opium poppy and *Thalictrum tuberosum*, respectively, whereas G3OMT had 64 % and 57% identity to reticuline 7OMTs from California poppy and opium poppy, respectively. On the other hand, G11OMT only had 35%, 42%, and 44% identity to flavonoid 7OMT from *Hordeum vulgare*, ipecac OMT from *Carapichea ipecacuanha* and 16-hydroxytabersonine OMT from *Catharanthus roseus*, respectively.

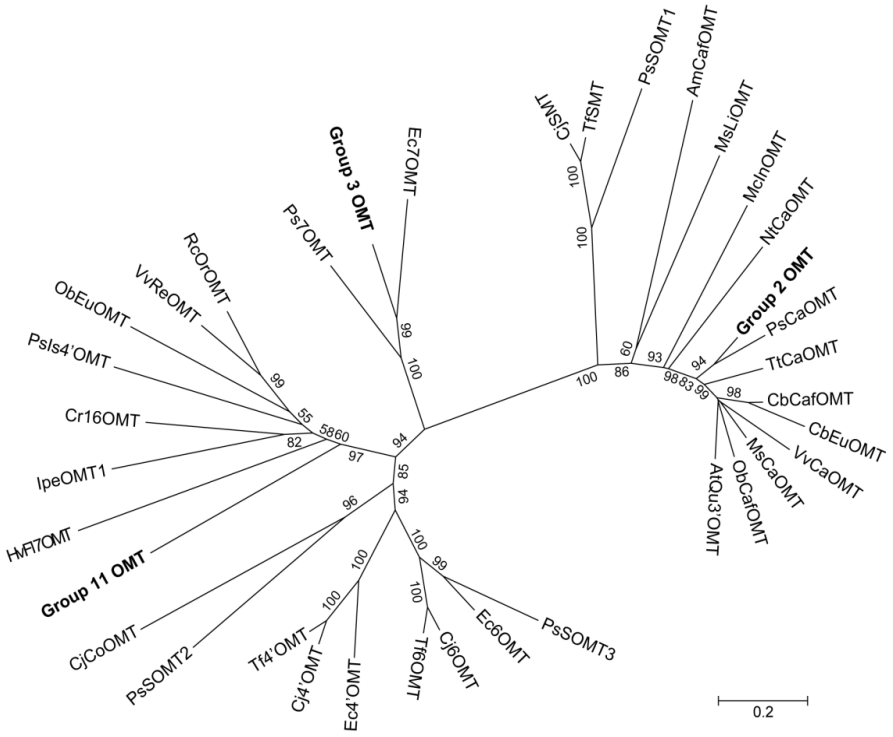


Figure 1-4. Unrooted neighbor-joining phylogenetic relationship of the three OMT candidates among known OMTs. The branch length is proportional to the estimated divergence distance of each protein. The scale bar (0.2) corresponds to a 20% change. The percentages of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches.

As the phylogenetic tree indicates (Figure 1-4), the three OMTs showed a distinct sequence identity from known OMTs in BIAs biosynthesis, and their distinct functions in the biosynthesis pathway in California poppy. G2OMT had 39% and 30%

identity with G3OMT and G11OMT, respectively, while G3OMT had 41% identity with G11OMT. Using BLAST searching, G3OMT also had a high identity (99% identity) to an uncharacterized putative *O*-methyltransferase (GenBank EU882970) registered by Liscombe et al., (2009).

Discussion

Many BIA biosynthesis pathways have been characterized at molecular level and considerable informations of biosynthetic enzymes and enzyme-coding genes on berberine, sanguinarine, morphine, and noscapine are accumulating (Ziegler and Facchini, 2008; Hagel and Facchini, 2013; Sato and Kumagai, 2013; Sato, 2013). The BIA biosynthesis in California poppy was also intensively examined, and most of BIA biosynthetic enzymes in California poppy were characterized at molecular level. However, the biosynthesis of several BIAs such as chelerythrine and macarpine were only partly characterized (Figure 0-1). A simple way to isolate the candidate genes is preparation of list of expressing enzyme genes and analysis of correlationship between accumulating metabolites and transcript accumulation. Using 22 candidate OMT genes and qRT-PCR analysis in 2 cell lines with different macarpine accumulation, G2, G3, and G11 genes were selected as a potential OMTs which might be involved in macarpine biosynthesis.

Phylogenetic analysis showed that these three OMTs were in different clades (Figure 1-4), indicating a distinct function in the biosynthesis pathway. Because sequence information and expression analysis were not sufficient to predict enzyme function, the full-length cDNAs of three candidate genes were isolated and expressed in *E. coli* cells (see Chapter 2).

Chapter II

Characterizations of OMT candidate in uncharacterized pathway in BIA biosynthesis

Introduction

As described in General Introduction, *O*-methyltransferases (OMTs) play important role in the biosynthesis pathway, including alkaloids, because *O*-methylation is crucial in directing intermediates to a specific pathway (Minami et al., 2008). Unique BIAs biosynthesis in California poppy started with berberine bridge enzyme (BBE, Dittrich and Kutchan, 1991), by which reticuline was converted to a closed-ring protoberberine alkaloid, scoulerine (Figure 0-1). Scoulerine was further converted to sanguinarine via methylene-ring formation by CYP719A5 (Ikezawa et al., 2009) and several additional enzymes such as CYP719A2, MSH, P6H, and DBOX (Ikezawa et al., 2007; Beaudoin and Facchini, 2013; Takemura et al., 2013; Hagel et al., 2012). Dihydrosanguinarine, precursor of sanguinarine, is also converted to macarpine by two uncharacterized P450s and OMTs and DBOX. On the other hand, chelerythrine, a BIA in California poppy, is produced by branch pathway via *O*-methylation of position 9 of scoulerine. Whereas biosynthetic enzymes in sanguinarine biosynthesis were characterized, biosynthetic enzymes involved in macarpine biosynthesis from dihydrosanguinarine or some enzymes in chelerythrine pathway from scoulerine were only partly characterized (Figure 0-1). In this chapter, I reported the characterizations of G3OMT gene, one of 3 candidate genes isolated in Chapter I.

After isolation of the full-length cDNA of OMT candidate genes (G2, G3, and G11), these cDNAs were cloned to *E. coli* using pET-21(d) expression vector to

produce recombinant proteins and the enzyme properties were characterized. Because only G3OMT was succesfully expressed in *E. coli* among the three OMTs, I focused on its enzymological characterization. Because G3OMT showed relatively high identity to reticuline 7OMT, enzyme activities were firstly examined with simple benzylisoquinolines. Because G3OMT showed unique dual *O*-methyltransferase activities, additional substrates were also examined. Finally, G3OMT was expressed as His-tagged protein and purified using Ni-resin column. Using purified G3OMT, enzyme substrate specificities and enzyme kinetics were further determined. Enzyme kinetics data suggested that scoulerine was the most preferential substrate for G3OMT, but G3OMT also methylated several substrates (reticuline, norreticuline, and scoulerine) in several positions and its biological role was not clear.

G3OMT was expected to be involved in multiple pathways in BIA biosynthesis, I evaluated the role of G3OMT in BIA biosynthesis using co-incubation of transgenic *Pichia* cells expressing several enzymes involved in BIA biosynthesis using pPIC3.5K expression vector. Co-incubation of enzymes with reticuline formed *N*-methylstylopine and *N*-methylcanadine. *O*-methylated reticuline formation was little. This result clearly suggests that G3OMT would have function as scoulerine 9-*O*-methyltransferase in the presence of BBE. Individual conversion experiment also showed that several novel metabolites could be produced from *O*-methylated reticulines and scoulerines, which were formed by G3OMT, suggesting that G3OMT can be useful bioconversion tool to produce new BIA compounds.

Materials and Methods

Chemicals

Following chemicals were used for substrate specificity analysis (Table 2-1). (*R*,*S*)-Reticuline, (*R*,*S*)-norreticuline, (*R*,*S*)-scoulerine, and (*R*,*S*)-6-*O*-methylnorlaudanosoline were from Mitsui petrochemical (Iwakuni, Japan). (*R*,*S*)-Laudanosoline was from Aldrich (Milwaukee, USA). 10-Hydroxychelerythrine was purified from California poppy S-38 cells using CombiFlash® (Teledyne Isco, Nebraska, USA). (*S*)-Tetrahydrocolumbamine was enzymatically prepared from (*S*)-scoulerine as described elsewhere (Ikezawa et al., 2003). Norlaudanosine was prepared from papaverine by chemical reduction with sodium borohydride. The purities of chemicals were confirmed by LC-MS and they were more than 91% pure.

2-[4-(Hydroxyethyl)-1-piperazinyl ethanesulfonic acid (HEPES), *N*-[tris(hydroxymethyl)methyl]glycine (tricine), and *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic (TAPS) acid were purchased from Dojindo (Kumamoto, Japan), whereas *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES), dipotassium hydrogenphosphate, and potasium dihydrogenphosphate were purchased from Nacalai tesque (Kyoto, Japan).

Effects of metal ions and chemicals on enzyme activity were examined with highest quality (> 99% purity) reagents of $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $CuSO_4.2H_2O$, $NiSO_4.6H_2O$, and iodoacetamide (IAA) purchased from Wako Pure Chemicals (Osaka, Japan). $ZnCl_2.3H_2O$ and $FeSO_4$ were obtained from Nacalai tesque (Kyoto, Japan).

AdoMet (*S*-adenosyl-L-methionine) was from BioLabs (England). β-mercaptoethanol, IPTG (isopropyl-β-D-thiogalactopyranoside), and polyacrylamide for

SDS-PAGE were from Nacalai tesque (Kyoto, Japan). Bradford reagent for protein quantification was from Bio-Rad (California, USA). Bovine serum albumin (BSA) was from Sigma (USA).

Expression of recombinant protein in Echerichia coli

The three full-length cDNAs of 3 OMT candidates (G2, G3, and G11) were cloned to the *BamHI* restriction site at the 5' and 3' ends in pET-21(d) expression vector (Novagen), then introduced to *E. coli* BL21 (DE3) (Novagen) as a host. Transgenic *E. coli* cells were grown in Luria Bertani (LB) medium (Invitrogen) at 200 rpm and 37^{o}C. After optical density (OD) at 600 nm reached 0.6-0.8, 1 mM IPTG was added and *E. coli* cells were further incubated at 16^{o}C for 24 hrs. The recombinant proteins were extracted from *E. coli* cells pellet, recovered by 3,300 x g centrifugation for 5 min, by the sonication in extraction buffer containing 100 mM potassium phosphate (pH 8.0), 10% glycerol, 5 mM β-mercaptoethanol and 5 mM sodium EDTA. After centrifugation at 15,300 x g for 20 min, the supernatants were desalted on PD10 column (GE Healthcare) and used as crude enzymes. Recombinant Ec7OMT was expressed as described elsewhere (Fujii et al., 2007).

Enzyme purification of G3OMT

For the preparation of purified G3OMT, I expressed G3OMT as His-tag protein and purified on Ni-resin based affinity chromatography. Six histidine tags were added to the 3' region of G3OMT and cloned to the *Nde I* and *Xho I* restriction sites at the 5' and 3' ends in pET-22(b) (Novagen). Expression vector was introduced in *E. coli* BL21 (DE3) (Novagen). After incubation in 2 mL LB medium at 25^{o}C, 200 rpm for overnight,

recombinant *E. coli* cells were inoculated in 600 mL LB medium and cultured at 37^{o}C, 200 rpm until OD_{600} reached 0.6-0.8. Recombinant protein induction was induced by the additional IPTG as described above. The cell extract was prepared as described above and applied to 10 mL Ni-affinity resin (Roche) column (Φ 12 mm) at flow rate 0.5 mL/min. Unabsorbed proteins were washed out with 80 mL buffer A (50 mM sodium phosphate buffer (pH 8.0) containing 400 mM NaCl), then His-tagged G3OMT was eluted with 15 mL buffer A containing 75 mM imidazole at flow rate 0.75 mL/min. The purified fractions were desalted on a PD-10 column, concentrated by Amicon Ultra-15 (Sigma) and stored in a solution of 100 mM potassium phosphate buffer (pH 7.2) with 40% glycerol until use. All purifications were performed at 4°C. The molecular mass of the enzyme was measured by SDS-PAGE (11.4% polyacrylamide). The protein concentration was determined by Bradford reagent with bovine serum albumin as the standard. Purity of purified protein was determined on SDS-PAGE by Image-J as 95.1% pure.

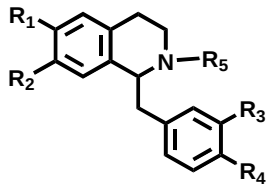
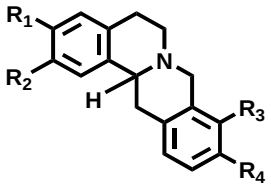
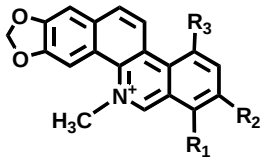
OMT assay

OMT activities were measured in 30 µL of 100 mM tricine buffer (pH 8.4) containing 10% glycerol, 5 mM β-mercaptoethanol, 5 mM sodium EDTA, 0.5 mM AdoMet, adequate substrate (100 µM) and the enzymes (ca. 50 µg crude protein or 5 µg purified protein) at 35°C in triplicates.

Preliminary analyses were done with crude enzyme and 60 min incubation with 100 µM substrate; simple benzylisoquinoline [(*R,S*)-laudanosoline, (*R,S*)-6-*O*-methylnorlaudanosoline, (*R,S*)-norreticuline, (*R,S*)-reticuline], a protoberberine ((*R,S*)-

scoulerine), and a benzophenanthridine alkaloid (10-hydroxychelerythrine). These analyses were confirmed with 5 μg purified enzyme in the same condition.

Table 2-1. Alkaloids used as substrate in enzyme assay

Alkaloid substrates		**R1**	**R2**	**R3**	**R4**	**R5**
	(*R*,*S*)-Laudanosoline	OH	OH	OH	OH	CH_3
	(*R*,*S*)-6-*O*-Methylnorlaudanosoline	OCH_3	OH	OH	OH	H
	(*R*,*S*)-Reticuline	OCH_3	OH	OH	OCH_3	CH_3
Simple benzylisoquinoline	(*R*,*S*)-Norreticuline	OCH_3	OH	OH	OCH_3	H
 Protoberberine	(*R*,*S*)-Scoulerine	OCH_3	OH	OH	OCH_3	-
 Benzophenanthridine	10-Hydroxychelerythrine	OCH_3	OCH_3	OH	-	-

Analysis of reaction time dependency was measured with 50 μg crude enzyme using 100 μM scoulerine as substrate, and this assay was confirmed using 5 μg purified enzyme. pH optimum, temperature optimum, and effects of chemicals were measured with 50 μg crude protein for 15 min using 100 μM scoulerine as substrate. For pH optimum assay, 100 mM buffer of HEPES, tricine, TAPS, CHES, and phosphates (K_2HPO_4 and KH_2PO_4) were used.

Assay for enyzme kinetics was performed in the optimum reaction conditions with 5 μg purified enzyme for 20 min using different concentration of reticuline,

norreticuline, and scoulerine as substrates in the presence of sufficient amount of AdoMet (0.5 mM).

Enzyme kinetics for AdoMet were also done with different concentration of AdoMet in the presence of sufficient amounts of reticuline (500 μM), norreticuline (100 μM) or scoulerine (100 μM). Enzymatic reactions were stopped by the addition of an equal volume of methanol containing 4% trichloroacetic acid and centrifugation at 15,300 x g for 20 min to remove proteins.

LC-MS analysis of reaction products

Reaction products were analyzed using an LC-MS 2020 (Shimadzu) with the following system: a TSKgel ODS-80 T_M column (4.6 mm i.d. × 250 mm, 5 μm, TOSOH, Japan), isocratic elution with solvent A (1% acetic acid) and solvent B (acetonitrile containing 1% acetic acid) with a composition of 30% solvent B for 20 min and flow rate of 0.6 mL/min at 40°C. When substrate 10-hydroxychelerythrine was used in enzyme assay, the solvent composition was 55% solvent B. The product formation was monitored by both the mass ion signal from 50 to 400 with electrospray ionization (ESI)-MS at 1.5kV (positive ion mode), and the UV spectrum at 190-600 nm measured by a photodiode array detector. MS fragment spectra of alkaloids were also analyzed by LC-MS 8030 (Shimadzu) system using same elution condition, ESI-MS with product ion scan mode, m/z 50.00-400.00, collision energy at -35.0V.

Reconstruction of biosynthetic pathway with recombinant proteins

To evaluate the physiological role of G3OMT and to test the possibility to produce novel compounds using G3OMT, I reacted *O*-methylation products of reticuline and scoulerine produced by G3OMT reaction with several biosynthetic enzymes in BIAs biosynthesis, which were expressed in *Pichia* cells with pPIC3.5K vector (Hori et al., 2016). The *O*-methylation products of reticuline and scoulerine were prepared by reaction of 200 μM (*R*,*S*)-reticuline and (*R*,*S*)-scoulerine with 100 μg G3OMT crude enzyme for 60 min in the condition as described in the OMT assay. Reaction products were recovered on Sep-Pak® column, the products were eluted with 3 mL methanol and evaporated to dryness. Recovered products were resolved in 50 μL DMSO and used as substrate for the successive reaction with *Pichia* cells.

Pichia cells were grown in 1 mL YPD medium (yeast extract, peptone, dextrose) for 24 hrs at 30^{o}C, then suspended in BMMY medium (buffered methanol-complex) and gene expression was induced by the addition of 0.5% methanol (final concentration). *Pichia* cells harboring BIAs biosynthesis enzymes were prepared as described elsewhere (Hori et al., 2016). After 24 hrs induction, 15 μL of substrate solution (*O*-methylated reticulines or scoulerines produced by G3OMT, 50 μM equivalent in total) was added, and further incubated for 48 hrs with 0.5% methanol addition at every 24 hrs.

To more directly evaluate the contribution of G3OMT in reticuline metabolism, I also incubated 200 μM (*R*,*S*)-reticuline with a mixture of BIA biosynthetic enzymes expressed in *Pichia* cells (G3OMT, BBE, CYP719A5, CYP719A2, CYP719A3, TNMT, and MSH). Canadine was also used as substrate for reaction with TNMT and

MSH to confirm the reaction. These two *Pichia* mixture systems were incubated in BMMY medium for 96 hrs at 30^{o}C with the addition of 0.5% methanol every 24 hrs.

Reaction products were extracted from *Pichia* cells in methanol containing 0.01 N HCl with sonication for 60 min, then analyzed by LC-MS/MS 8030 (Shimadzu) as described above.

Results

Expression of recombinant proteins in E. coli cells and its purification

Expression vector pET-21(d) and *E. coli* BL21 were used to produce recombinant protein to analyse the enzymological properties of G2, G3, and G11 OMTs. As Figure 2-1 shows, only G3OMT showed successful expression of 40 kD recombinant protein as soluble form. G2 and G11 did not show any visible recombinant proteins in soluble fraction. G11 showed only degraded protein accumulation in insoluble fraction (Figure 2-1A). Whereas I tried to express G2 and G11 protein in *E. coli* at different conditions, all trials were failed. Therefore, I focused on the characterization of G3OMT. After the enzyme assay with crude G3OMT, purified G3OMT was also prepared using 3'end- His-tagged G3OMT. His-tagged G3OMT was successfully purified on a Ni-NTA column and an ImageJ analysis showed that purified G3OMT was 95.1% pure (Figure 2-1B). When enzyme activities of G3OMT with His-tag and without tag were compared, little difference of enzyme activity was detected on protein basis (data not shown), whereas a little changes in α helix of protein stucture may affect the His-tagged activity (Panek et al., 2013).

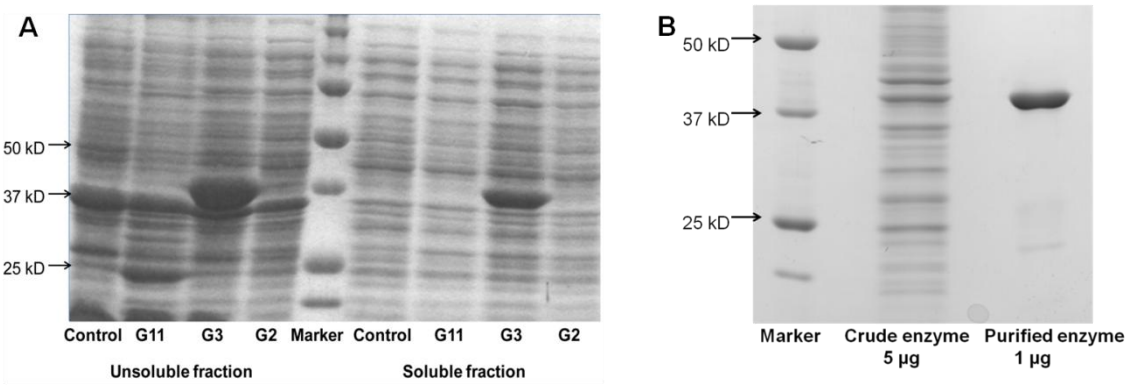


Figure 2-1. Expression of candidate OMTs in *E. coli* after IPTG induction for 24 hrs. Ten ng protein was applied on SDS-PAGE.
(A) The expression of G2, G3, and G11 OMT using expression vector pET-21(d)
(B) The expression of His-tagged G3OMT and purification by Ni-NTA column

OMT activity

G3OMT activity was evaluated first with simple benzylisoquinoine alkaloid, reticuline, because G3OMT had high identity with *E. californica* 7OMT (Figure 1-4) (Ec7OMT, Fujii et al., 2007). Then, some related BIAs to reticuline were examined. First assay was done with crude enyzme (Figure 2-2) and the results were further confirmed with purified enzyme (Figure 2-3). When G3OMT activity was determined with reticuline, G3OMT had an activity to methylate 7 and 3' positions of reticuline to produce laudanine (m/z 344) and codamine (m/z 344), respectively (Figure 2-2A, Figure 2-3A), whereas Ec7OMT only methylated reticuline at 7- position to produce laudanine (Figure 2-2B). Interestingly, G3OMT also fully methylated reticuline and produced laudanosine (m/z 358).

Reaction products were determined by mass fragmentation analysis in comparison with to reticuline standard that showed a m/z 192 (isoquinoline moiety) and m/z 137 (benzyl moiety) fragments. An increase in 14 m/z of isoquinoline moiety (from m/z 192 to m/z 206) indicating 7-*O*-methylation to produce laudanine, whereas increase in 14 m/z of benzyl moiety (from m/z 137 to m/z 151) indicating 3'-*O*-methylation to

produce codamine. On the other hand, increases in 14 m/z in both isoquinoline and benzyl moieties indicated both methylation of 7 and 3' positions and production of laudanosine (Figure 2-4A).

G3OMT also methylated norreticuline (another simple benzylisoquinoline) with a similar pattern to reticuline; G3OMT produced 7-*O*- and 3'-*O*-methylation products (norlaudanine and norcodamine, respectively), and dual methylation product, norlaudanosine (Figure 2-2C, Figure 2-3B). On the other hand, Ec7OMT only produced mono-methylation products for norreticuline (Figure 2-2D). Because norreticuline do not have *N*-methylation in benzylisoquinoline structure, its m/z value of isoquinoline moiety is 178. Accordingly, increases in 14 m/z of isoquinoline moeity (from m/z 178 to m/z 192) or benzyl moiety (from m/z 137 to m/z 151), indicated 7 or 3'-*O*-methylation to produce norlaudanine and norcodamine, respectively. Increases in 14 m/z of both isoquinoline and benzyl moieties indicated the production of norlaudanosine (Figure 2-4B). The capability of G3OMT to produce norlaudanosoline (tetrahydropapaverine), indicated that G3OMT might be involved in the formation of papaverine under certain conditions.

Because G3OMT showed broad enzyme activities, I examined other BIA substrates, such as a protoberberine, scoulerine. When G3OMT reacted with scoulerine, G3OMT methylated scoulerine at 9 and 2 positions to produce tetrahydrocolumbamine (m/z 342) and 2-*O*-methylscoulerine (m/z 342) as single methylation products (Figure 2-2E). G3OMT also showed dual methylation activity with scoulerine to produce tetrahydropalmatine (m/z 356) (Figure 2-2E, Figure 2-3C). When Ec7OMT reacted with scoulerine, Ec7OMT also methylated scoulerine but produced 2-*O*-methylscoulerine (Figure 2-2F).

These reaction products were also determined by their fragmentation patterns in comparison with standards of scoulerine, tetrahydrocolumbamine and tetrahydropalmatine (Figure 2-4C). Scoulerine has m/z 328, whereas scoulerine isoquinoline moiety has m/z 178. The fragmentation pattern of tetrahydrocolumbamine (m/z 342) produced ion fragment with m/z 178, and means that methylation occured in benzyl moiety, not in isoquinoline moiety, whereas in case of 2-*O*-methylscoulerine (m/z 342), its detected ion fragment was m/z 192, and means that a methylation occured in isoquinoline moiety (from m/z 178 to m/z 192). On the other hand, tetrahydropalmatine (m/z 356), its detected fragment ion was 192, indicated methylation occured both in isoquinoline and benzyl moieties.

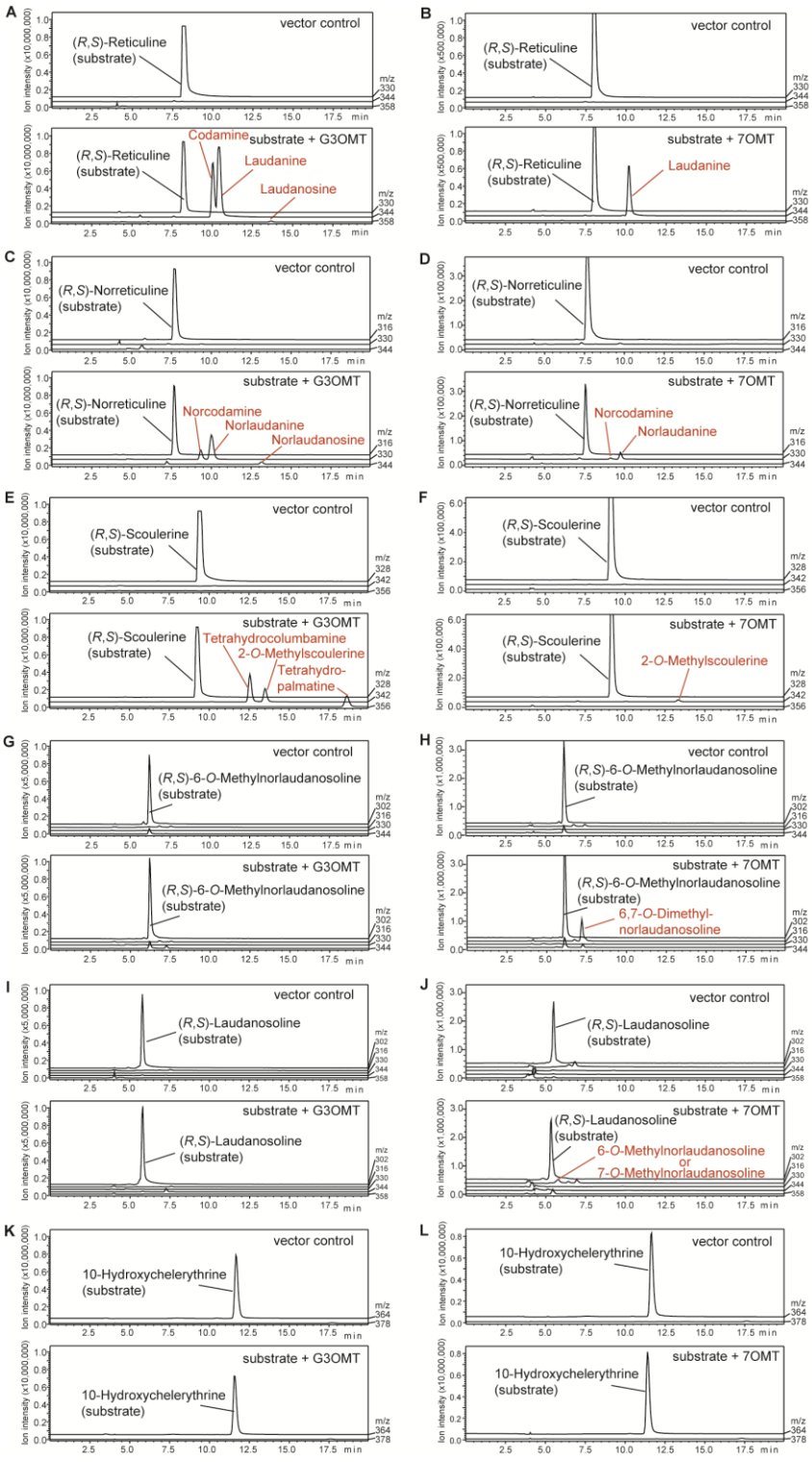


Figure 2-2. LC-MS analyses of enzyme reaction products by crude G3OMT (A, C, E, G, I, K) or Ec7OMT (B, D, F, H, J, L). Reactions were done with reticuline (A, B), norreticuline (C, D), scoulerine (E, F), 6-*O*-methylnorlaudanosoline (G, H), laudanosoline (I, J), and 10-hydroxychelerythrine (K, L). The red lines indicate reaction products. Reactions A, C, E were confirmed by purified enzyme (Figure 2-3).

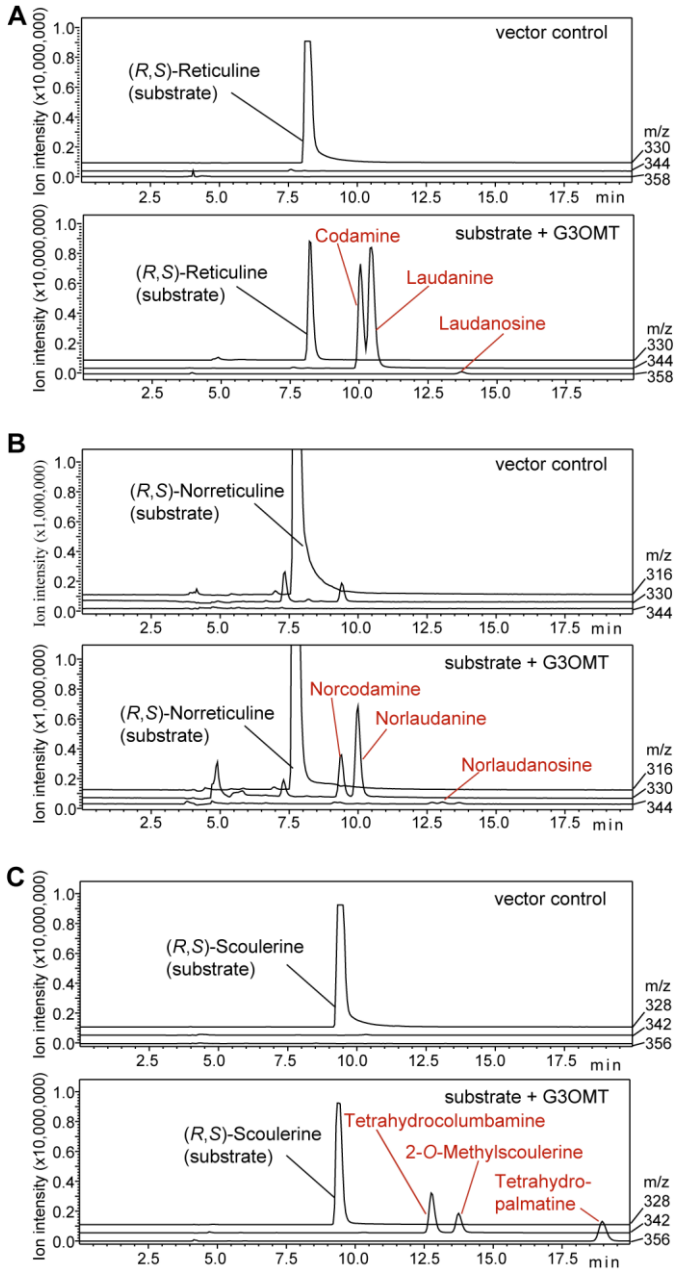


Figure 2-3. LC-MS analyses of enzyme reactions products by purified G3OMT. Reactions were done with reticuline (A), norreticuline (B), and scoulerine (C). Mass ion signals were determined with LC-MS/MS 8030 and monitored with selected ion monitoring mode as described in Materials and Methods. The red lines indicate reaction products.

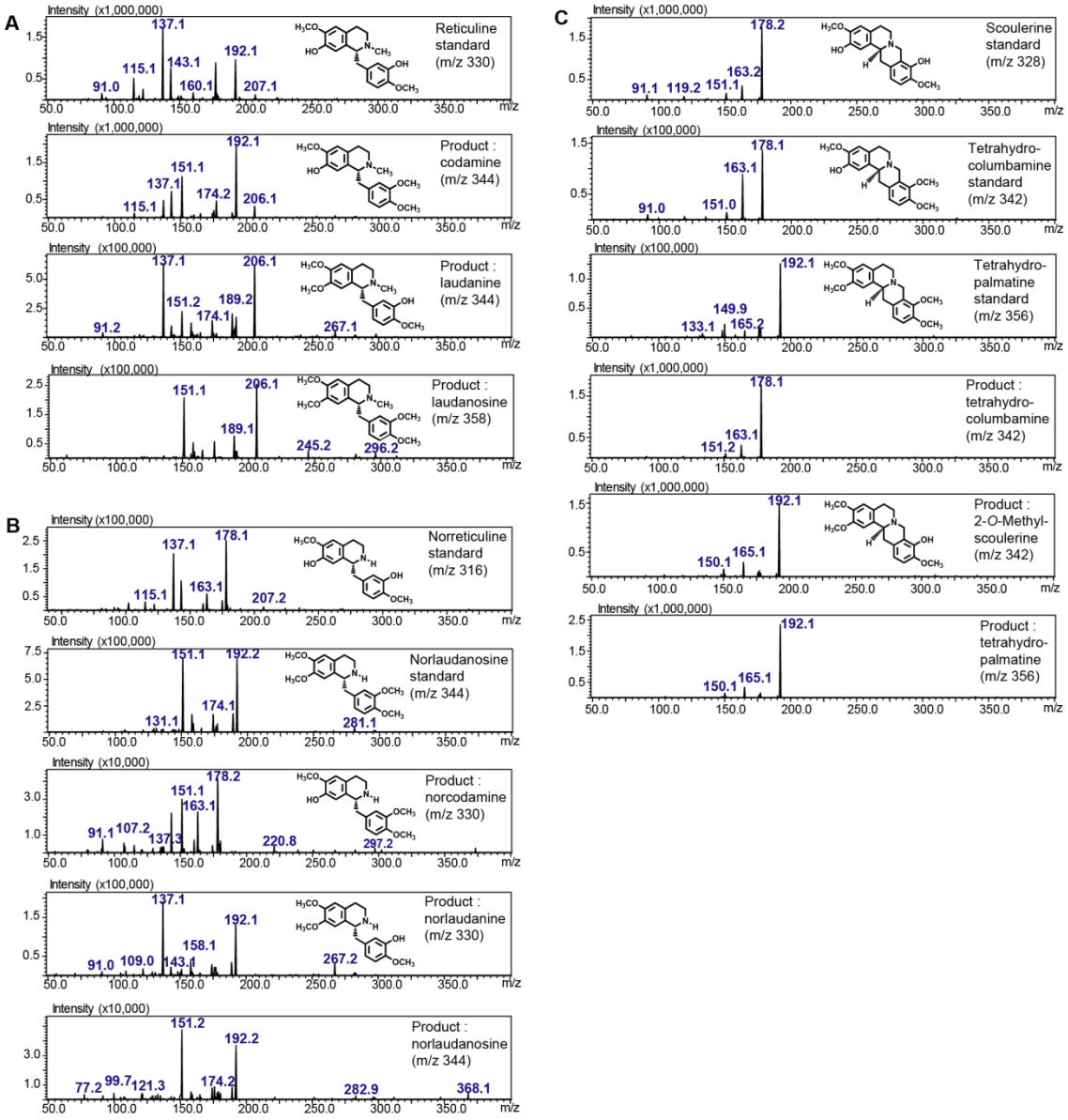


Figure 2-4. Product annotation based on the fragmentation pattern of *O*-methylated reticuline (A), norreticuline (B), and scoulerine (C).
The reaction products produced in Figure 2-3 were analyzed with a LC-MS/MS 8030 (Shimadzu) coupled with a triple-quadrupole mass analyzer operating in positive ion mode with an electrospray ionization (ESI) source at a collision energy of -35 V.

When G3OMT reacted with other simple benzylisoquinolines (6-*O*-methylnorlaudanosine and laudanosine), G3OMT showed no activity (Figure 2-2 G,I), whereas Ec7OMT produced mono-methylated product, 6,7-*O*-dimethylnorlaudanosine, from 6-*O*-methylnorlaudanosoline (Figure 2-2H), and produced 7-*O*-methylation

product (either 6-*O*-methyllaudanosoline or 7-*O*-methyllaudanosoline was not confirmed) from laudanosine (Figure 2-2J, Figure 2-5). On the other hand, when a benzophenanthridine alkaloid (10-hydroxychelerythrine) was reacted, neither G3OMT nor Ec7OMT methylated this compound (Figure 2-2K,L).

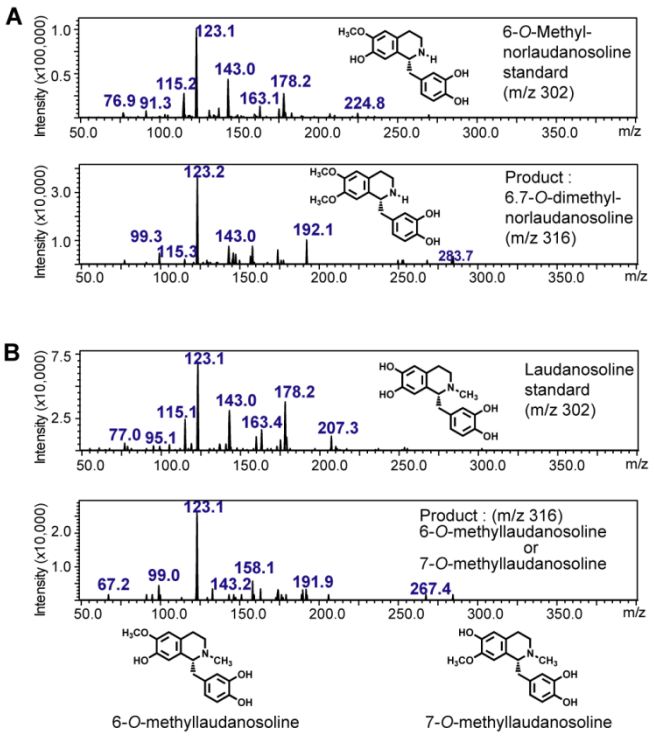


Figure 2-5. Determination of *O*-methylated products produced by Ec7OMT from 6-*O*-methylnorlaudanosoline (A), or laudanosoline (B). Mass fragmentation pattern were analyzed by LC-MS/MS 8030 (Shimadzu).

Because G3OMT showed dual *O*-methylation activities for two hydroxy groups of reticuline or scoulerine, these reaction kinetics were analyzed within shorter incubation times. Short incubation clearly indicated the single methylation product formation by G3OMT. In the case of reticuline, or norreticuline, 7-*O*-methylation (laudanine and norlaudanine) were more preferential than 3'-*O*-methylation products (codamine and norcodamine). In the case of scoulerine, 9-*O*-methylation (tetrahydrocolumbamine) was more preferential than 2-*O*-methylation (2-*O*-

methylscoulerine). This reaction specificity was obviously different from scoulerine *O*-methyltransferase 1 (PsSOMT1) of *P. somniferum*. PsSOMT1 had activity to mono-methylate reticuline or scoulerine to produce codamine or tetrahydrocolumbamine, then sequentially produced dual *O*-methylated products from reticuline and scoulerine (Dang and Facchini, 2012).

The G3OMT abilities to produce several metabolites from reticuline, norreticuline, and scoulerine suggested that G3OMT would have an important role in the regulation of BIA pathway in California poppy and its biological role was examined below.

Enzyme properties

Before the detailed enzymological characterization of G3OMT, some enzyme properties were examined. First, incubation time dependency of enzyme reaction was examined. Crude enzyme G3OMT showed linear product formations during 5-15 min for 100 μM reticuline, norreticuline, or scoulerine (Figure 2-6A), whereas purified G3OMT showed linear product formations during 10-25 min for same amount of reticuline, norreticuline, or scoulerine (Figure 2-6B).

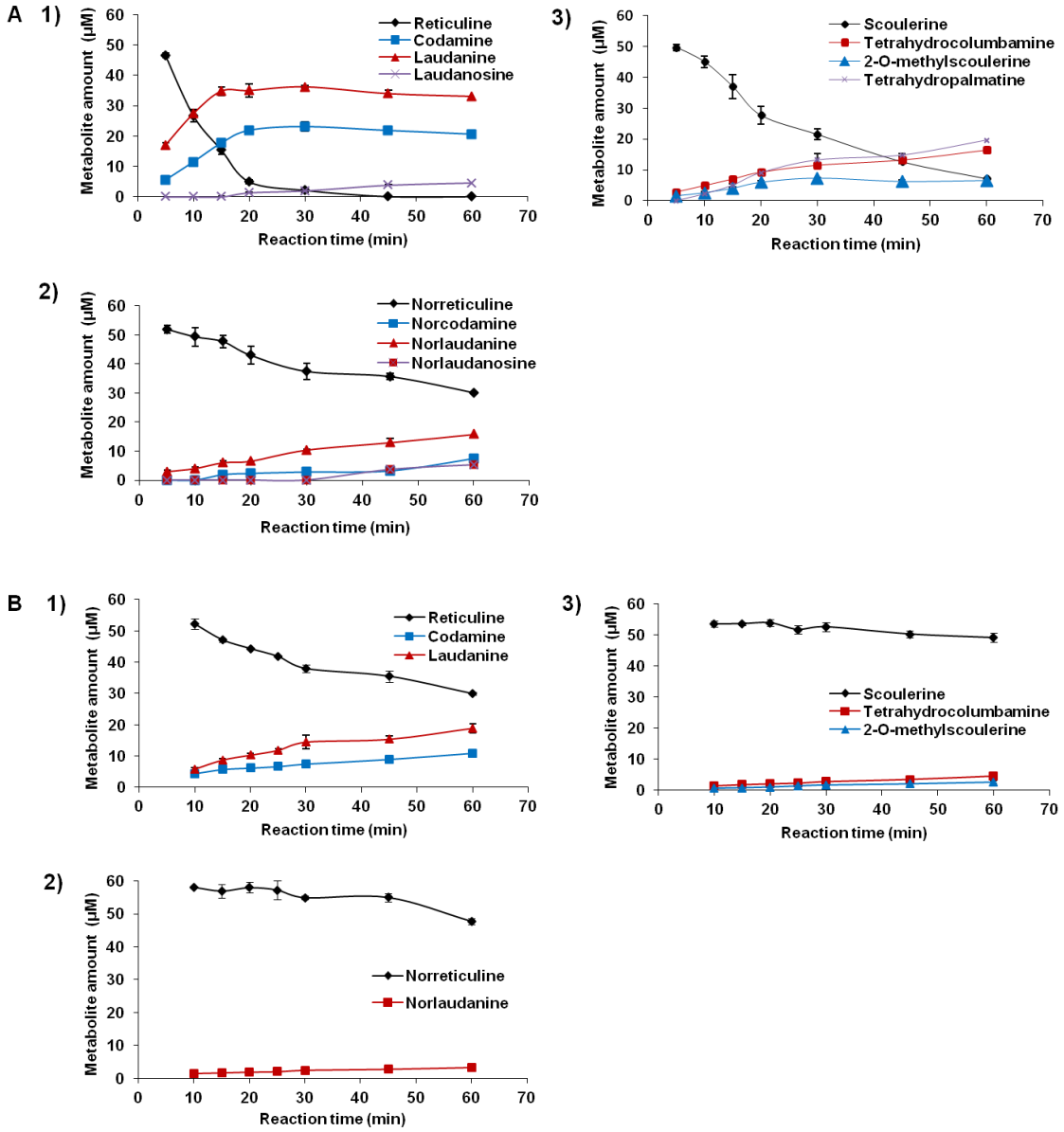


Figure 2-6. G3OMT activities against reticuline (1), norreticuline (2), and scoulerine (3) using crude (A) or purified (B) enzymes. Each value represents the mean ± SD of three replicates. Reaction products were analyzed by LC-MS 2020 (Shimadzu).

Because G3OMT showed unique activity to produce mono and dual methylated products with scoulerine, pH optimum, temperature optimum, and effects or chemicals were examined with crude enzyme using scoulerine as substrate. G3OMT showed broad pH optimum ranging from pH 6.8 to 9.6, and its highest activity was in pH 8.4 in tricine buffer (Figure 2-7). Both 9-*O*-methylation and 2-*O*-methylation activities showed similar pH optimum. This optimum pH was slightly higher than Coptis 4' OMT (pH 8.0) (Morishige et al., 2000) but lower than 6OMT (pH 9.0) (Sato et al., 1994). Thus, enzyme kinetic measurement was done with tricine buffer at pH 8.4 (Figure 2-7).

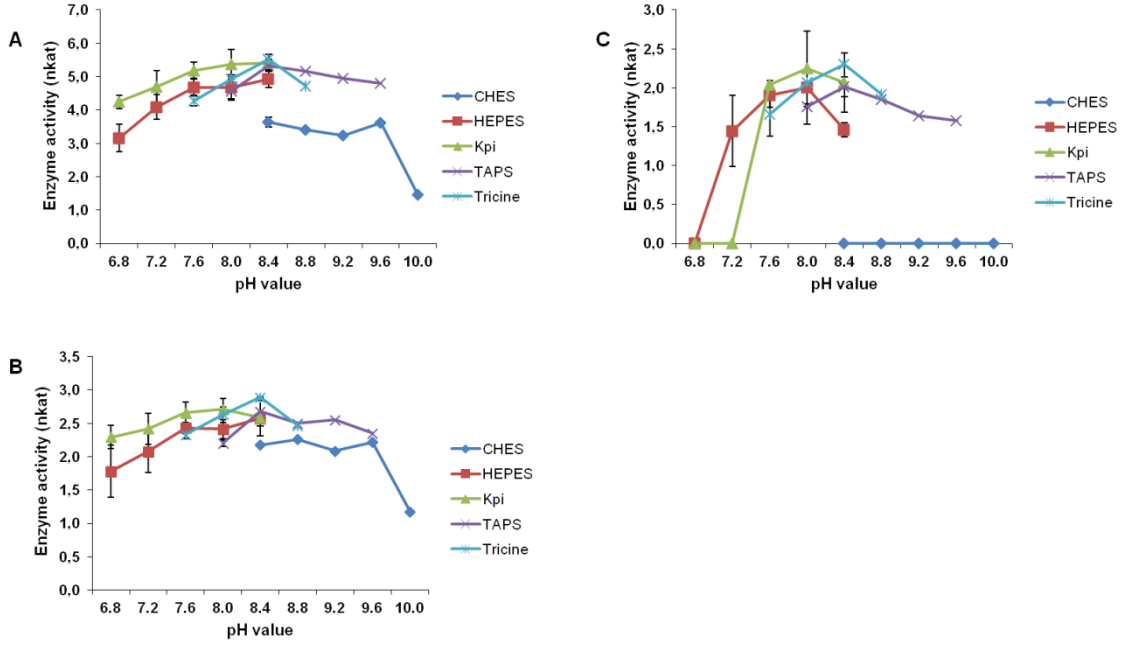


Figure 2-7. Optimum pH of G3OMT reaction with scoulerine as substrate. The formation of tetrahydrocolumbamine (A), 2-*O*-methylscoulerine (B), and tetrahydropalmatine (C) were determined. Each value represents the mean ± SD of three replicates.

Optimum temperature was also determined with scoulerine. G3OMT showed the highest activity at 35°C for both 9-*O*-methylation and 2-*O*-methylation (Figure 2-8).

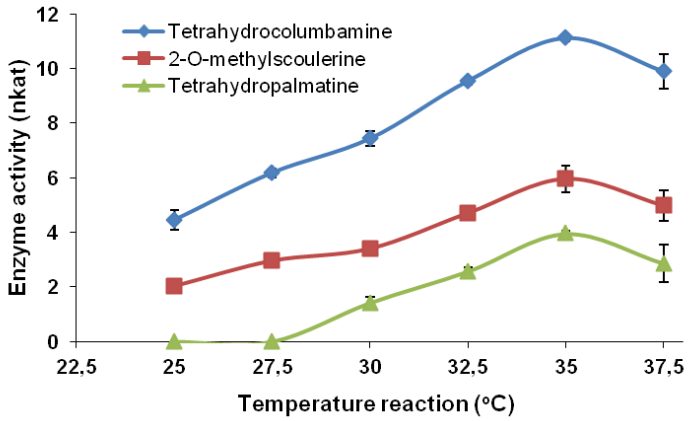


Figure 2-8. Optimum temperature of G3 reaction for scoulerine. The formation of tetrahydrocolumbamine, 2-*O*-methylscoulerine, and tetrahydropalmatine were determined. Each value represents the mean ± SD of three replicates.

Effects of chemicals on G3OMT activity were also examined with 100 µM scoulerine as a substrate. G3OMT did not need divalent cations for its activity (Figure 2-9). Mg^{2+}, Mn^{2+}, Fe^{2+}, and iodoacetamide at 5 mM also did not inhibit G3 activity. Ca^{2+}, Co^{2+}, and β-mercaptoethanol showed slight inhibition of 7%, 9%, and 9%, respectively. On the other hand, Cu^{2+}, Ni^{2+}, and Zn^{2+} showed inhibition of 21%, 18%, and 20%, respectively (Figure 2-9A). When 100 µM palmatine or berberine (protoberberines) were added in the enzyme reaction, they did not inhibit G3OMT activity. But when 20 µM chelerythrine or sanguinarine (benzophenanthridine alkaloids) were added, they inhibited 59 and 87% of G3OMT activity (Figure 2-9B). Inhibition of OMT activity by the addition of sanguinarine was also reported for Coptis 6OMT (Sato et al., 1994) or Tf6OMT (Robin et al., 2016).

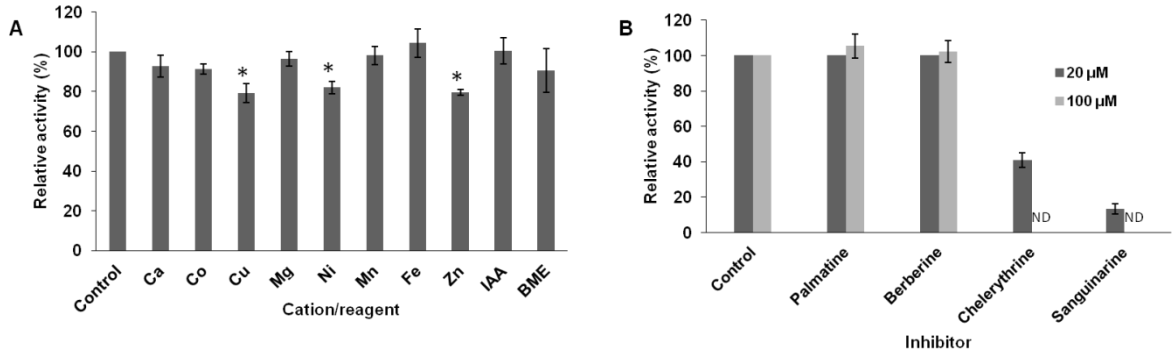


Figure 2-9. Effects of metal ions and some chemicals on G3 OMT activity.
All tested cations are bivalent. Each value represents the mean ± SD of three replicates. * indicates the statistical significance by Student's t-test at $p<0.05$. Abbreviations: IAA, iodoacetamide; BME, β-mercaptoethanol. ND: not detected

Enzyme kinetics

Under the optimized reaction conditions, G3OMT enzyme kinetics were examined using purified enzyme with sufficient amount 0.5 mM of methyl donor (AdoMet) (Figure 2-10, Table 2-2). When reticuline was used as substrate, methylation of position 7 (laudanine formation) was more prefentially than codamine formation at all examined concentration. When norreticuline was used, only formation of norlaudanine (7-*O*-methylation) was detected and this methylation was slower than 7-*O*-methylation of reticuline. When scoulerine was reacted with G3OMT, formation of tetrahydrocolumbamine (9-*O*-methylation) was more preferential than that of 2-*O*-methylscoulerine (2-*O*-methylation) at all tested concentrations. The dose dependency curves of the substrates showed that scoulerine was the most reactive among the three substrates (Figure 2-10).

Kinetic analyses with substrates showed that reactions followed the Michaelis-Menten model (Figure 2-10). When reticuline was the substrate, the K_m values for the formation of laudanine (7-*O*-methylation) and codamine (3'-*O*-methylation) were 393 µM and 187 µM and the k_{cat}/K_m values were 0.61 and 0.27 s^{-1} mM^{-1}, respectively. In the

case of norreticuline, the K_m value of norlaudanine formation was 38.2 µM, and the k_{cat}/K_m value was 0.26 s^{-1} mM^{-1}. In the case of scoulerine, the K_m values for the formation of tetrahydrocolumbamine and 2-*O*-methylscoulerine were 24.5 and 21.9 µM, and the k_{cat}/K_m values were 0.82 and 0.46 s^{-1} mM^{-1}, respectively (Table 2-2).

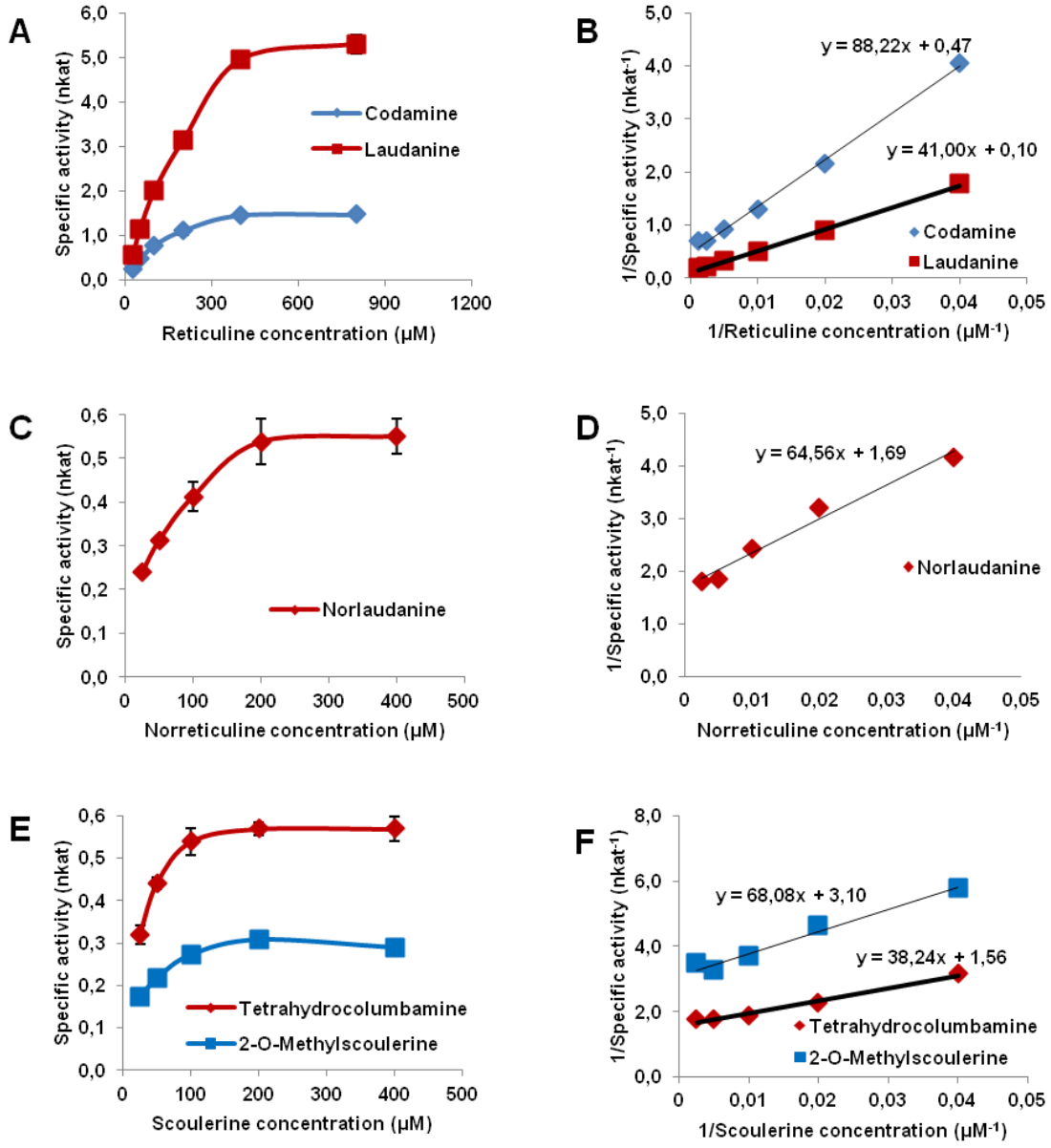


Figure 2-10. Michaelis-Menten and Lineweaver-Burk plots of G3OMT against reticuline (A and B), norreticuline (C and D), and scoulerine (E and F).
Each value represents the mean ± SD of three replicates. Reaction products were analyzed by LC-MS 2020 (Shimadzu).

The enzyme kinetics of G3OMT for AdoMet (methyl donor) were examined with each substrate using sufficient amounts of each alkaloid substrate (Figure 2-11). When reticuline was used as substrate, the K_m values of AdoMet for the formation of laudanine and codamine were 119 μM and 14.5 μM, while the k_{cat}/K_m values were 1.00 and 5.51 s^{-1} mM^{-1}, respectively. When norreticuline was used as substrate, the K_m value and k_{cat}/K_m of AdoMet for the formation of norlaudanine were 15.7 μM and 0.64 s^{-1} mM^{-1}. In the case of scoulerine as substrate, the K_m values of AdoMet for the formation of tetrahydrocolumbamine and 2-*O*-scoulerine were 9.3 μM and 28.8 μM, whereas the k_{cat}/K_m values were 1.07 and 0.35 s^{-1} mM^{-1}, respectively (Table 2-2).

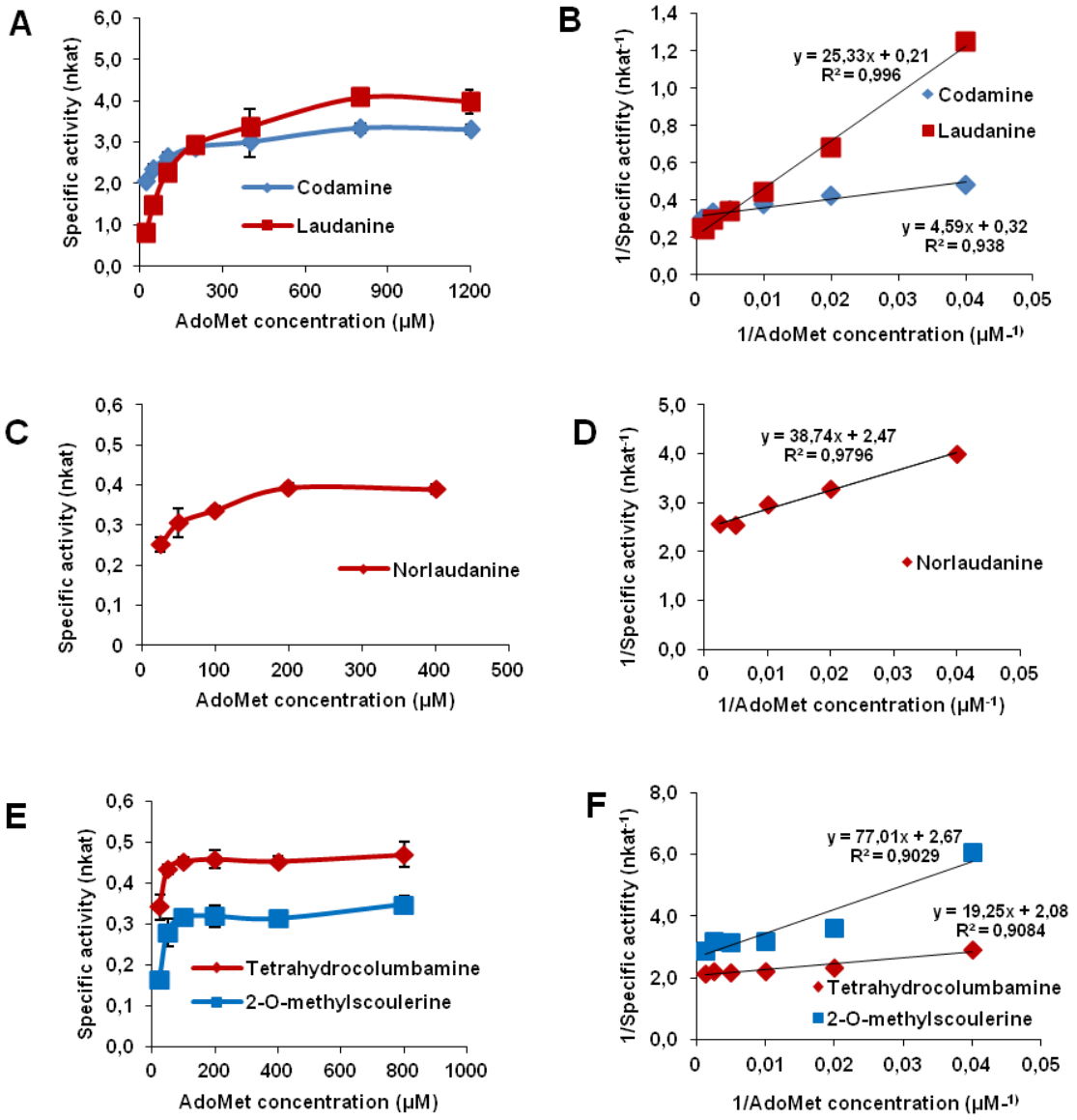


Figure 2-11. AdoMet dose dependence curve of G3OMT (A, C, E) and their Lineweaver-Burk plots (B, D, F) for substrate, 500 μM reticuline (A and B), 100 μM norreticuline (C and D), or 100 μM scoulerine (E and F).
Each value represents the mean ± SD of three replicates. Reaction products were analyzed by LC-MS 2020 (Shimadzu).

Table 2-2. Enzyme kinetic data for several scoulerine-*O*-methyltransferases

Enzyme	Substrate	Product	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.mM^{-1}$)	Ref.
PsSOMT1	Scoulerine	Tetrahydrocolumbamine	28.5 ± 6.8	1.44 ± 0.27	50.5	Dang and Facchini (2012)
	AdoMet		19 ± 2.7	0.91 ± 0.001	47.89	
	Reticuline	Codamine	70.3 ± 13.7	0.13 ± 0.07	1.85	
PsSOMT2	Scoulerine	Tetrahydrocolumbamine	73.3 ± 18.6	0.09 ± 0.013	1.23	
	AdoMet		69.62 ± 20.9	0.09 ± 0.04	1.25	
PsSOMT3	Scoulerine	Tetrahydrocolumbamine	50.8 ± 13.6	0.06 ± 0.01	1.25	
	AdoMet		101.2 ± 29.4	0.07 ± 0.02	0.71	
CjSMT	Scoulerine	Tetrahydrocolumbamine	100.0	2.25	22.5	Takeshita et al. (1995)
	AdoMet					
G3OMT	Scoulerine	Tetrahydrocolumbamine	24.5	0.02	0.82	This study
		2-*O*-methylscoulerine	21.9	0.01	0.46	
	AdoMet	Tetrahydrocolumbamine	9.3	0.01	1.07	
		2-*O*-methylscoulerine	28.8	0.01	0.35	
	Reticuline	Codamine	187	0.05	0.27	
		Laudanine	393	0.24	0.61	
	AdoMet	Codamine	14.5	0.08	5.51	
		Laudanine	119.0	0.12	1.00	
	Norreticuline	Norlaudanine	38.2	0.01	0.26	
	AdoMet		15.7	0.01	0.64	

Estimation of the biosynthetic role of G3OMT in BIAs biosynthesis using co-culture of Pichia cells expressing BIA enzymes

Enzymological properties of G3OMT suggested that G3OMT catalyzes the *O*-methylation of scoulerine in the chelerythrine biosynthesis. To examine this hypothesis, I re-constructed biosynthetic pathway using several enzymes in BIA biosynthesis, such as : BBE (Dittrich and Kutchan, 1991), cheilanthifoline synthase (CYP719A5, Ikezawa et al., 2009), stylopine synthase (CYP719A2/A3, Ikezawa et al., 2007), stylopine *N*-

methyltransferase (TNMT, Liscombe and Facchini, 2007), and *N*-methylstylopine hydroxylase (MSH, Beaudoin and Facchini, 2013) using *O*-methylation products of reticuline and scoulerine produced by G3OMT as substrate.

First, I reacted the *O*-methylated reticulines. When BBE, which catalyzed the oxidative cyclization of *N*-methyl moiety into the berberine bridge carbon and converted reticuline into scoulerine (Kutchan and Dittrich, 1995), was reacted with *O*-methylated reticulines produced by G3OMT (i.e. codamine, laudanine, and laudanosine), only laudanine was converted by BBE to 2-*O*-methylscoulerine, whereas codamine and laudanosine were not (Figure 2-12A). It suggested that an *ortho*-methoxyphenol in the benzyl moiety was important to form a closed ring of tetrahydroberberine. On the other hand, CYP719A5 and CYP719A2 did not react with reticuline or its *O*-methylated products, which indicated the importance of a berberine bridge ring for the reactions.

Although G3OMT could react with reticuline to produce laudanine and codamine *in vitro*, these metabolite products did not detected in California poppy cell cultures. It suggested that reticuline was not a substrate of G3OMT *in vivo*. Whereas the mechanism how G3OMT did not react with reticuline *in vivo* was not clear, the separation of biosynthetic enzymes by sieve elements or cell compartment may regulate the of alkaloid biosynthesis, as proposed by Amann et al., 1986; Ziegler and Facchini, 2008 or strong activity of BBE may compete G3OMT for reticuline and excrude G3OMT from the reaction as shown below.

When *O*-methylated scoulerines produced by G3OMT were reacted with either CYP719A5, CYP719A2, or CYP719A3, several products in BIAs biosynthesis were detected (Figure 2-12B). Tetrahydrocolumbamine, a major *O*-methylated product of

scoulerine by G3OMT, was converted to canadine (m/z 340) by CYP719A3, and further converted by TNMT to *N*-methylcanadine (m/z 354), a precursor of allocryptopine in the chelerythrine biosynthesis (Figure 2-13). On the other hand, CYP719A2 did not show any product against *O*-methylated scoulerines. These results indicated the different functional role of CYP719A2 and CYP719A3 in BIAs biosynthesis; CYP719A2 in sanguinarine biosynthesis and CYP719A3 in chelerythrine biosynthesis. Scoulerine itself was converted to nandinine (m/z 326) by CYP719A2 and CYP719A3.

When *O*-methylated scoulerines were reacted with TNMT, several *N*-methylation products were detected (Figure 2-12B). Scoulerine was *N*-methylated to be *N*-methylscoulerine (m/z 342), tetrahydrocolumbamine was converted to *N*-methyltetrahydrocolumbamine (m/z 356), and tetrahydropalmatine was converted to *N*-methyltetrahydropalmatine (m/z 370), whereas 2-*O*-methylscoulerine was not *N*-methylated by TNMT. *O*-methylated scoulerines also did not react with MSH.

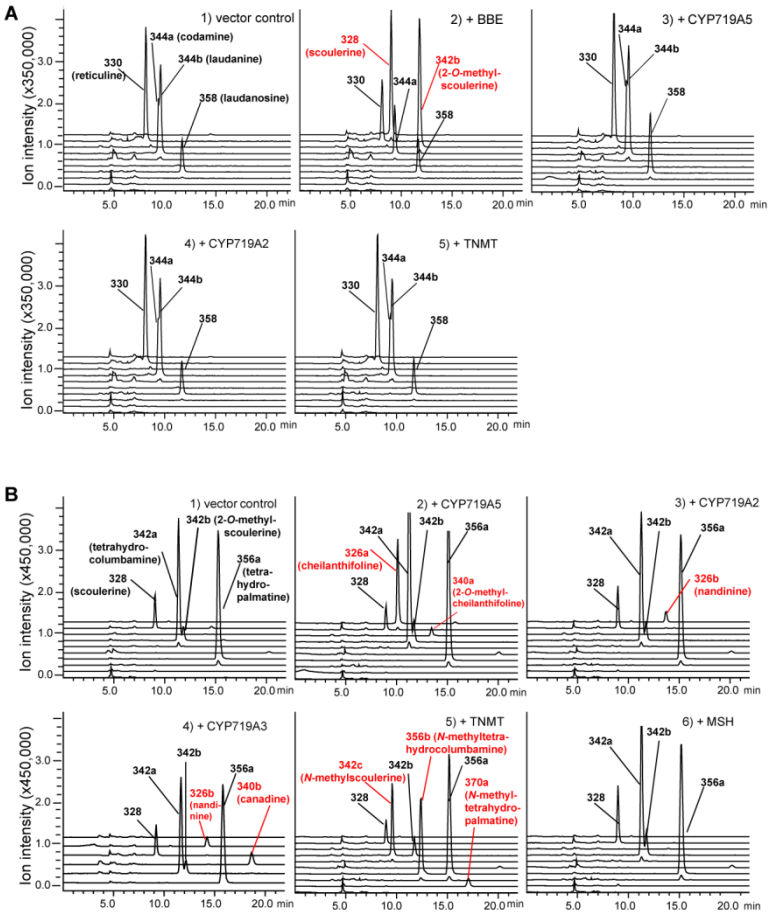


Figure 2-12. Bioconversion of *O*-methylated reticulines (A), and *O*-methylated scoulerines (B) by biosynthetic enzyme in BIAs biosynthesis pathway which was expressed in *Pichia pastoris* GS-115 harboring pPIC3.5K expression vector. Enzyme used in A was 1) vector control, 2) BBE, 3) CYP719A5, 4) CYP719A2, or 5) TNMT. Enzyme used in B was 1) vector control, 2) CYP719A5, 3) CYP719A2, 4) CYP719A3, 5) TNMT, or 6) MSH. Reaction products were assigned using fragmentation pattern and/or authentic standard.

Determined products : m/z 326a, cheilanthifoline; m/z 326b, nandinine; m/z 328, scoulerine; m/z 330, reticuline; m/z 340a, 2-*O*-methylcheilanthifoline; m/z 340b, canadine; m/z 342a, tetrahydrocolumbamine; m/z 342b, 2-*O*-methylscoulerine; m/z 342c, *N*-methylscoulerine; m/z 344a, codamine; m/z 344b, laudanine; m/z 356a, tetrahydropalmatine; m/z 356b, *N*-methyltetrahydrocolumbamine; m/z 358, laudanosine; m/z 370a, *N*-methyltetrahydropalmatine, respectively. Black peaks are starting materials for bioconversion; whereas red peaks indicate the new products formed by added enzyme.

Abbreviations: BBE, Berberine Bridge Enzyme; CYP719A2, stylopine synthase, CYP719A3, stylopine/canadine synthase; CYP719A5, cheilanthifoline synthase; MSH, *N*-methylstylopine 14-hydroxylase; TNMT, tetrahydroberberine *N*-methyltransferase; *N*-methyl THC, *N*-methyltetrahydrocolumbamine; *N*-methyl THP, *N*-methyl-tetrahydropalmatine.

Estimation of biosynthetic role of G3OMT in BIA biosynthesis using co-culture of Pichia cells expressing multiple biosynthetic enzymes

To examine biological role of G3OMT in reticuline metabolism, all biosynthetic enzymes in reticuline metabolism in BIA biosynthesis were co-incubated with reticuline. In fact, BIA enzymes (G3OMT, BBE, CYP719A5, CYP719A2, CYP719A3, G3OMT, TNMT and MSH), were expressed in *Pichia* cells (Hori et al., 2016) and co-cultured with reticuline as substrate. As shown in Figure 2-13, reticuline was converted to *N*-methylstylopine in sanguinarine pathway and *N*-methylcanadine in chelerythrine pathway. *N*-methylstylopine was most abundant, but considerable amounts of *N*-methylcanadine were detected. Whereas *O*-methylated scoulerines were detected adequately, only small amounts of *O*-methylated reticulines were detected. This result showed that BBE very actively reacted with reticuline to produce scoulerine and reaction of reticuline with G3OMT was little, indicating that reticuline *in vivo* was not substrate of G3OMT. Also, this result indicated BBE reaction product, scoulerine, was efficiently converted by CYP719A5, CYP719A2, then TNMT to cheilanthifoline, stylopine, and then *N*-methylstylopine. Similarly, scoulerine was converted by G3OMT, CYP719A3, TNMT, and MSH to tetrahydrocolumbamine, canadine, *N*-methylcanadine, and allocryptopine. This result strongly suggests that G3OMT functions as scoulerine 9-*O*-methyltransferase *in vivo* in chelerythrine biosynthesis.

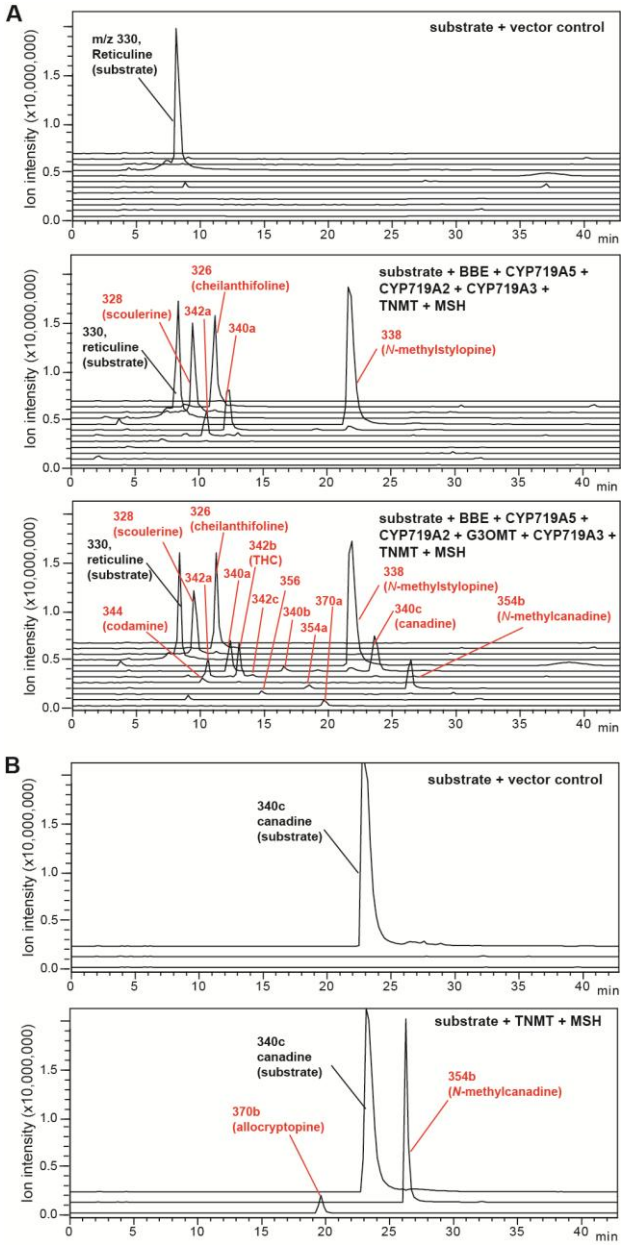


Figure 2-13. LC-MS analyses of co-incubation products of reticuline (A) or canadine (B) with biosynthetic enzymes involved in BIAs biosynthesis. Reticuline was co-incubated with BBE, CYP719A5, CYP719A2, CYP719A3, TNMT, MSH, and G3OMT. Protopine was not detected in this reaction. The reaction products were assigned by LCMS/MS 8030.

Reaction products: m/z 326, cheilanthifoline; m/z 328, scoulerine; m/z 330, reticuline; m/z 338, *N*-methylstylopine, m/z 340a, *N*-methylcheilanthifoline; m/z 340b, 2-*O*-methylcheilanthifoline; m/z 340c, canadine; m/z 342a, *N*-methylscoulerine, m/z 342b, tetrahydrocolumbamine; m/z 342c, 2-*O*-methylscoulerine; m/z 344, codamine; m/z 354a, *N*-methyl-2-*O*-methylcheilanthifoline; m/z 354b, *N*-methylcanadine; m/z 356, *N*-methyltetrahyrocolumbanine; m/z 370a, *N*-methyltetrahydropalmatine; m/z 370b, allocryptopine, respectively. Black colors indicate the starting materials for reaction, and the red colors indicate the products.

Discussion

In this research, I identified enzyme activity of G3 *O*-methyltransferase isolated from California poppy. G3OMT showed OMT activities for scoulerine (a protoberberine), reticuline, and norreticuline (simple benzylisoquinolines), and methylated both hydroxyl moieties of those substrates (Figure 2-2, Figure 2-3). Although G3OMT shared high identity to Ec7OMT and Ps7OMT (64 and 57% identity, respectively), G3OMT showed distinct methylation activity from them. Ec7OMT and Ps7OMT only methylated reticuline at 7 position to form laudanine (Fujii et al., 2007; Ounaroon et al., 2003), whereas G3OMT methylated reticuline at 7 and 3' positions to form laudanine and codamine, respectively. For scoulerine, Ec7OMT only produced low amount of 2-*O*-methylscoulerine, and Ps7OMT gave no product (Ounaroon et al., 2003), whereas G3OMT actively showed dual *O*-methylation activity and converted scoulerine to mono methylated products, tetrahydrocolumbamine and 2-*O*-methylscoulerine, and dual methylated product, tetrahydropalmatine. Whereas, another scoulerine OMT with some reaction similarity was isolated from *P. somniferum* (PsSOMT1), its function was not in chelerythrine biosynthesis, but in noscapine and papaverine biosynthesis (Dang and Facchini, 2012). PsSOMT1 also produced 3'-*O*-methylation product, codamine, from reticuline, but PsSOMT1 was in the different clade to Ec7OMT (Figure 1-4) and distinct reaction properties from G3OMT.

Whereas enzyme properties such as high k_{cat}/K_m values and high affinity (small K_m) of G3OMT to scoulerine suggested the role of G3OMT as scoulerine OMT, its broad substrate specificities suggest its broad biological role in BIA biosynthesis. Reverse-genetic approach is common to determine the physiological role, I tried to use synthetic biological way to evaluate this biosynthetic enzyme. When G3OMT was co-

incubated with *Pichia* cells expressing several BIA biosynthesis enzymes, G3OMT showed clear production of canadine or *N*-methylcanadine from reticuline via tetrahydrocolumbamine, which were further converted to allocryptopine by CYP719A3, TNMT, and MSH (Figure 2-13). Thus, G3OMT was estimated as the missing scoulerine OMT involved in chelerythrine biosynthesis and related alkaloids (Figure 0-1).

To understand the broad reaction specificity of G3OMT (Table 2-2), comparison of G3OMT with 7OMT with relatively high sequence identity would be useful. As mentioned above, G3OMT methylated scoulerine at 9 and 2 positions and reticuline at 7 and 3' positions, whereas Ec7OMT only methylated reticuline and norreticuline at 7 position and scoulerine at 2 position. Ps7OMT only methylated reticuline at 7 position and no activity against norreticuline and scoulerine (Ounaroon et al., 2003). It is important to note that Ec7OMT activity was only detected in high accumulation of reticuline in plant cell (Fujii et al., 2007). Similarly, G3OMT activity other than scoulerine 9-*O*-methylation might not occur under normal physiological conditions, in which no inhibition of other biosynthetic enzyme and substrate reticuline accumulation occurs.

About the biological role of G3OMT, one of puzzling questions is the low expression level of G3OMT in S-38 cells with high 10-hydroxychelerythrine but high in the A5-1 cells with high macarpine (Figure 1-1). If G3OMT functions as scoulerine OMT in S-38 cells in chelerythrine pathway, G3OMT should be expressed as high as A5-1 cells. Whereas low expression in S-38 cells suggest that G3OMT was little involved in chelerythrine biosynthesis, S-38 cells were transformant expressing Coptis *SMT* gene. Therefore, high expression of Coptis *SMT* gene was expected to down-regulate the endogeneous *G3OMT* gene expression, then compensate the chelerythrine

biosynthesis. This unique phenomenon would be an interesting subject for further investigations.

On the other hand, it is also interesting to know how scoulerine OMT activity was obtained in G3OMT, even though the sequence identity of G3OMT to Papaver SOMT1 and Coptis SMT was only 40%. Other Papaver SOMTs, PsSOMT2 and PsSOMT3, which also directing on 9-*O*-methylation of scoulerine, only shared 36% identity to G3OMT. This rather large sequence differences among scoulerine OMTs suggest that scoulerine *O*-methyltransferase may obtain their activity independently during evolution. This finding also explains why the scoulerine *O*-methyltransferase gene in California poppy was not detected when searched by Coptis SMT sequence (Takemura et al., 2010a).

Although the amino acid sequences of scoulerine OMTs and G3OMT were different (Figure 1-4), G3OMT and PsSOMT1 have several similarities, such as activity to the substrates (scoulerine, reticuline, and norreticuline) and the dual regio-specific reactivities. On the other hand, the 3D-structure of enzyme with substrate was necessary to characterize molecular mechanism, especially how G3OMT simultaneously methylates scoulerine at 9 and 2 position, whereas PsSOMT1 only methylates scoulerine at 9 position and then position 2 in the successive methylation.

G3OMT amino acid sequence with other OMTs in BIA biosynthesis

Each *O*-methyltransferase has a conserved sequence for AdoMet binding, which consists of several motif sequence (Kagan and Clarke, 1994; O'Gara et al., 1995; Struck et al., 2012). Among these motifs, motif I (9 amino acids), motif II (8 amino acids), and motif III (10 amino acids), are more characterized than others (Joshi and Chiang, 1998).

Recently, Robin et al., (2016) reported the crystal structure of *Thalictrum flavum* 6OMT (Tf6OMT) with its substrate, norlaudanosoline. Crystal structures of isoflavone *O*-methyltransferase (IOMT) and chalcone *O*-methyltransferase (ChOMT) from *Medicago sativa* were also reported (Zubieta et al., 2001). Unfortunately, the sequence identity of those three OMTs to G3OMT was not so high. Tf6OMT shared 38%, 31%, 39%, 59%, 33%, 39%, 39%, 42%, and 32% sequence identity to G3OMT, PsSOMT1, PsSOMT2, PsSOMT3, CjSMT, Ec7OMT, Ps7OMT, IOMT, and ChOMT, respectively. IOMT shared 39%, 27%, 37%, 35%, 30%, 37%, 38%, 42%, and 28% identity to G3OMT, PsSOMT1, PsSOMT2, PsSOMT3, CjSMT, Ec7OMT, Ps7OMT, Tf6OMT, and ChOMT, respectively. On the other hand, ChOMT shared 36%, 37%, 29%, 29%, 38%, 33%, 35%, 32%, and 28% identity to G3OMT, PsSOMT1, PsSOMT2, PsSOMT3, CjSMT, Ec7OMT, Ps7OMT, Tf6OMT, and IOMT, respectively. Their 3D-structural information, however, would be useful for molecular characterization of G3OMT function, especially the conserved of AdoMet binding domain and general secondary structure.

In Tf6OMT, AdoMet binding site was estimated with the co-crystal formation of AdoMet homologue, *S*-adenosyl-L-homocysteine (SAH). The SAH-binding residues showed conserved in OMT sequences (G in motif I, D in motif II, D in motif III, and K

in motif IV (Figure 2-14). The catalytic domain (H) estimated by SAH-binding was also conserved in those OMTs. As I discussed above, G3OMT, PsSOMT, and CjSMT showed scoulerine OMT activity and G3OMT, Ec7OMT, and Ps7OMT showed reticuline 7OMT activities beside other enzyme activities. Unfortunately, the sequence diversification of these OMTs is too large except conserved AdoMet binding sites or catalytic residues. Currently I am conducting crystal formation of G3OMT and hope to reveal the secret of broad reactivity of G3OMT in near future using crystal structure of G3OMT.

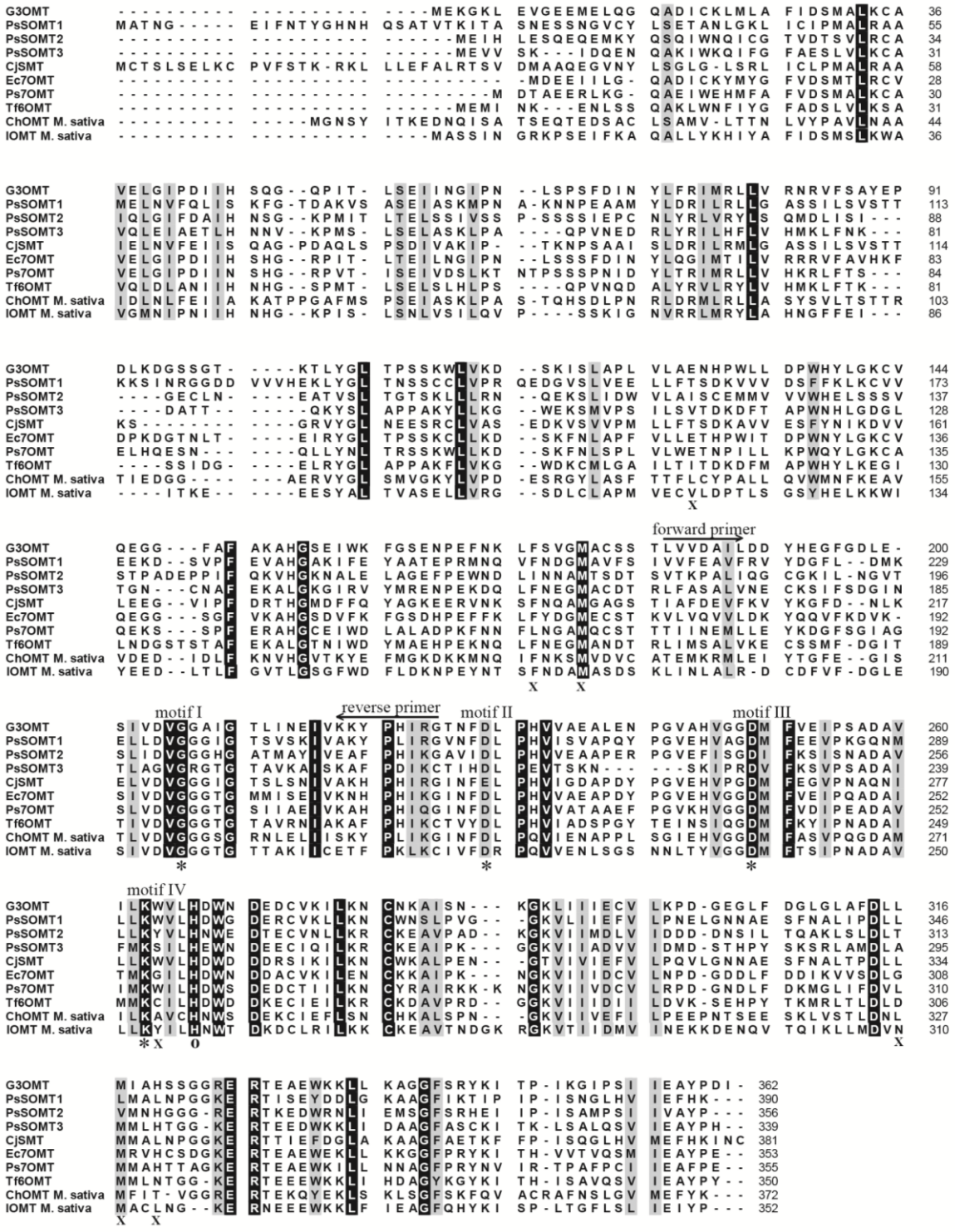


Figure 2-14. Amino acid sequence alignment of G3OMT and other characterized OMTs. Conserved amino acid of all sequences are highlighted in black, while similar sequences are shaded. Based on the conserved function according to the crystal structure of TfOMT, IOMT, and ChOMT, “asterik” is AdoMet binding domain, “circle” is catalytic residue, and “cross” is substrate binding residue of IOMT, Tf6OMT, and ChOMT. Forward and reverse primers used for RT-PCR in Table 1-1 were designed as shown.

The broad enzyme activity of G3OMT would be biotechnologically applicable

When G3OMT reacted with reticuline or scoulerine and their *O*-methylated products were further used for bioconversion by biosynthetic enzymes in BIA pathway, many novel products were detected (Figure 2-12, Figure 2-13, Figure 2-15). The broad activity of G3OMT is surely useful for pathway re-construction for novel alkaloid production in microbes (Minami et al., 2008; Hori et al., 2016) and open the new field of BIA bioproduction.

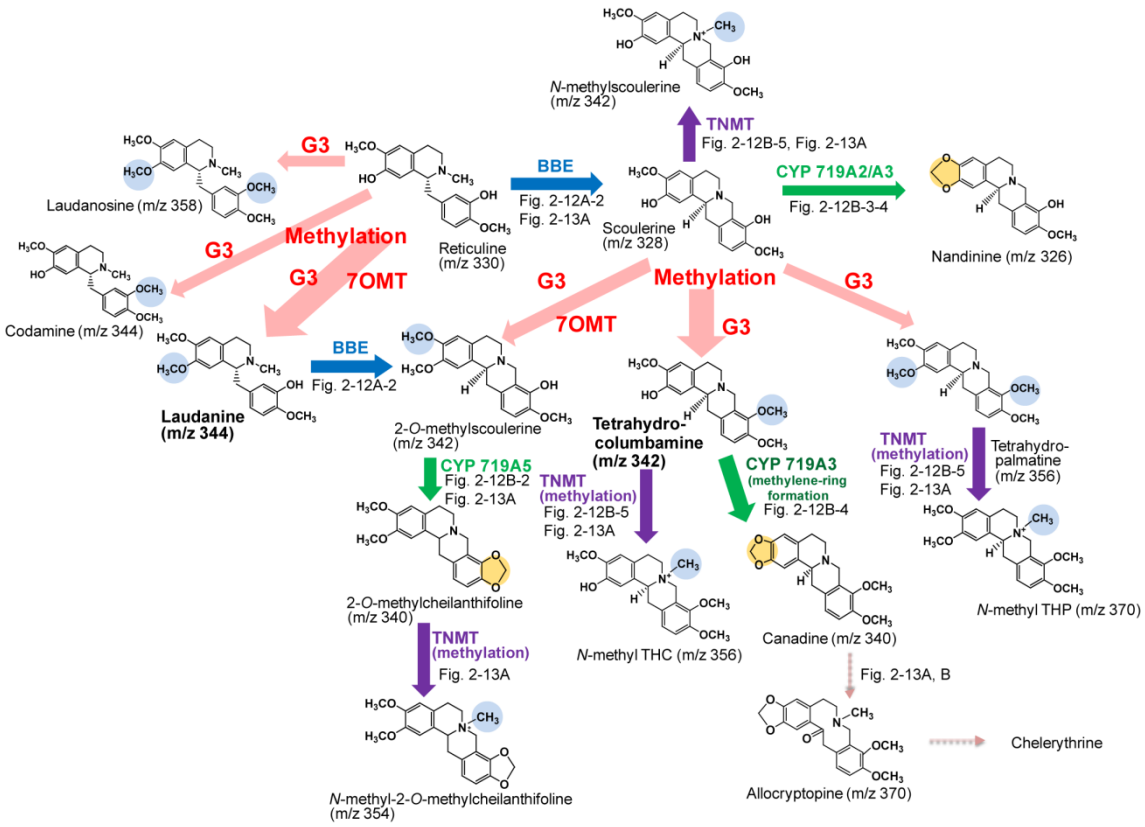


Figure 2-15. Novel BIA production by bioconversion of reticuline and scoulerine by G3OMT and several enzyme involved in BIAs biosynthesis. Figure is redrawn based on the results of Figure 2-12 and Figure 2-13.

Summary and Perspectives

Chapter I

O-methyltransferases (OMTs) are a large enzyme family to *O*-methylate hydroxyl moiety of many natural products using *S*-adenosyl-L-methionine (AdoMet) as methyl donor. OMT is also important to direct a specific and certain reaction in plant specialized metabolism. Because OMTs have a conserved AdoMet binding domain, many OMT sequences have been so far isolated and characterized using this conserved sequence as a query for screening.

In this study, I attempted to isolate uncharacterized biosynthetic enzyme-coding genes in benzylisoquinoline alkaloid (BIA) biosynthesis in California poppy using the OMT signature sequence. First, using several sequences of known OMTs in BIAs biosynthesis (4'OMT, 6OMT, and 7OMT from California poppy; 4'OMT, 6OMT, SMT, and CoOMT from *Coptis japonica*), many OMT-like sequences were found from California poppy transcriptome data in the NCBI and PhytoMetaSyn. Based on the sequence similarities of their conserved binding domain, 68 uncharacterized OMT-like sequence were grouped into 22 groups and the longest clones were selected as uncharacterized OMT candidates. To select the candidate genes involved in uncharacterized BIA biosynthesis, such as macarpine or chelerythrine, the gene expression of each candidate gene was determined by quantitative RT-PCR using cDNAs of California poppy cells with different macarpine/10-hydroxyxhelerythrine production; a low macarpine but high 10-hydroxychelerythrine producing S-38 and a high macarpine producing A5-1 cells. Three OMT candidates (G2, G3, G11) were selected by the high expression in A5-1 cells. The full-length cDNAs of three OMTs were prepared and their sequences were compared with other known OMTs.

Phylogenetic tree clearly showed that those three OMTs were in different clades and might have distinct function in the BIA pathway.

Chapter II

To characterize enzyme properties and role of candidate OMTs in BIAs biosynthesis, the full-length cDNA of *G2*, *G3*, and *G11OMT* were isolated and their recombinant proteins were expressed in *E. coli* BL21 (DE3). Among those three OMTs, only G3OMT was successfully expressed, and its enzymological properties were further characterized after the purification of enzyme.

When G3OMT activities were examined with reticuline, G3OMT showed dual *O*-methylation activity at both positions 7 and 3', whereas Ec-reticuline 7OMT (64% identity with G3OMT), showed mono-methylation at position 7. G3OMT also produced dual *O*-methylated laudanosine from reticuline. Similarly, G3OMT showed dual *O*-methylation activity for *N*-demethylated norreticuline, whereas enzyme activity was lower than that for reticuline. Whereas G3OMT had low sequence identity to known scoulerine 9OMT, G3OMT also methylated scoulerine at 9 and 2 positions to produce tetrahydrocolumbamine and 2-*O*-methylscoulerine, and also dual *O*-methylated tetrahydropalmatine. Because G3OMT had broad activities for BIA substrates, G3OMT was expected to be involved in various BIA biosynthesis.

When enzyme kinetics of G3OMT were determined, G3OMT showed lower *Km* value for scoulerine than other substrates, such as reticuline and norreticuline. But *Kcat/Km* values of G3OMT from scoulerine to tetrahydrocolumbamine was rather moderate in comparison with that from reticuline to laudanine.

Therefore, the biological role of G3OMT in BIAs biosynthesis was evaluated using co-culture of *Pichia* cells expressing G3OMT and several biosynthetic enzymes in BIAs biosynthesis, such as BBE, CYP719A5, CYP719A2, CYP719A3, TNMT, and MSH, and reticuline as substrate. This co-culture system with reticuline successfully reconstructed the biosynthetic pathway in BIA biosynthesis and produced key intermediates *N*-methylstylopine and *N*-methylcanadine in sanguinarine and chelerythrine biosynthesis, respectively. These results strongly suggested that G3OMT would function as scoulerine-9-*O*-methyltransferase in chelerythrine biosynthesis and BBE is critical enzyme in the reticuline metabolism. This co-incubation experiment also showed the high potential of G3OMT to produce diversified metabolites in BIA biosynthesis.

Perspectives

In this research, I attempted to isolate several uncharacterized enzymes in BIA biosynthesis from California poppy, especially in involved in macarpine biosynthesis, as mentioned in the introduction in Chapter II. From my searched OMTs, I found 3 OMT candidate genes, G2, G3, and G11OMT. Unfortunately, only G3OMT was successfully expressed as recombinant protein. Further characterization of G3OMT showed that G3OMT was identified as scoulerine OMT in chelerythrine biosynthesis. Therefore, my first attempt to isolate OMT candidate involved in macarpine biosynthesis is still on going. In fact, in the case of G11OMT, recombinant protein was formed in insoluble fraction and degraded. More optimization of heterologous expression was needed. When I tried to express G11OMT in yeast, G11OMT showed enzyme activity to methylate 10-hydroxydihydrosanguinarine in macarpine biosynthesis

(unpublished result). Therefore, more optimization of heterologous expression was needed as well as reverse genetic approaches to characterize the *in vivo* function of candidate enzymes.

Whereas I could not conduct metabolic engineering of BIA biosynthesis with isolated OMT candidate genes, pathway engineering using overexpression of key enzymes, down regulation of pathway enzymes, and overexpression of general transcription factors would be promissing. Because plant cell systems have more potentials for the production of metabolites and diversity, metabolic engineering would be still very promissing technique to produce desired metabolites.

On the other hand, I used synthetic approach to characterize the G3OMT function using reticuline, and *O*-methylated reticulines and scoulerines produced by G3OMT, with several enzymes in BIA biosynthesis, which were expressed in *Pichia* cells. The results showed that G3OMT prefered to *O*-methylate scoulerine than reticuline to produce tetrahydrocolumbamine, a precursor of chelerythrine, in the presence of BBE. It suggested that such synthetic approach would be useful to predict the role of G3OMT in plant cells. These results also showed several novel metabolites produced by this *Pichia* system, which are not presence in nature. G3OMT would be useful as bioconversion tool in synthetic biology of BIA biosynthesis.

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Purwanto

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