Unraveling Additional O-Methylation Steps in Benzylisoquinoline Alkaloid Biosynthesis in California Poppy (Eschscholzia californica)

Ratmoyo Purwanto, Kentaro Hori, Yasuyuki Yamada and Fumihiko Sato*

Laboratory of Molecular and Cellular Biology of Totipotency, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo, Kyoto 606-8502, Japan

*Corresponding author: E-mail, fsato@lif.kyoto-u.ac.jp; Fax, +81-75-753-6398.

(Received April 4, 2017; Accepted June 30, 2017)

California poppy (Eschscholzia californica), a member of the Papaveraceae family, produces many biologically active benzylisoquinoline alkaloids (BIAs), such as sanguinarine, macarpine and chelerythrine. Sanguinarine biosynthesis has been elucidated at the molecular level, and its biosynthetic genes have been isolated and used in synthetic biology approaches to produce BIAs in vitro. However, several genes involved in the biosynthesis of macarpine and chelerythrine have not yet been characterized. In this study, we report the isolation and characterization of a novel O-methyltransferase (OMT) involved in the biosynthesis of partially characterized BIAs, especially chelerythrine. A search of the RNA sequence database from NCBI and PhytoMetaSyn for the conserved OMT domain identified 68 new OMT-like sequences, of which the longest 22 sequences were selected based on sequence similarity. Based on their expression in cell lines with different macarpine/chelerythrine profiles, we selected three OMTs (G2, G3 and G11) for further characterization. G3 expression in Escherichia coli indicated O-methylation activity of the simple benzylisoquinolines, including reticuline and norreticuline, and the protoberberine scoulerine with dual regio-reactivities. G3 produced 7-O-methylated, 3'-O-methylated and dual O-methylated products from reticuline and norreticuline, and 9-O-methylated tetrahydrocolbambine, 2-O-methylscoulerine and tetrahydropalmatine from scoulerine. Further enzymatic analyses suggested that G3 is a scoulerine-9-O-methyltransferase for the biosynthesis of chelerythrine in California poppy. In the present study, we discuss the physiological role of G3 in BIA biosynthesis.

Keywords: Benzylisoquinoline alkaloids • Biosynthetic enzyme • California poppy • Eschscholzia californica • O-Methyltransferase • Scoulerine.

Abbreviations: AdoMet, S-adenosylmethionine; BBE, berberine bridge enzyme; BIA, benzylisoquinoline alkaloid; CNMT, coclairine N-methyltransferase; CoOMT, cumbarine O-methyltransferase; CYP7192A3, stylopine synthase; CYP719AS, cheilanthifoline synthase; DBOX, dihydrobenzophenanthridine oxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; MSH, N-methylstylopine 14-hydroxylase; OMT, O-methyltransferase; 4’OMT, N-methylcoclaurine 4’-O-methyltransferase; 6OMT, norcooclaurine 6-O-methyltransferase; PGH4, protopine 6-hydroxylase; SMT, scoulerine 9-O-methyltransferase of Coptis japonica; SOMT, scoulerine 9-O-methyltransferase in noscapine biosynthesis of Papaver somniferum; TNMT, tetrahydropseudoberberine N-methyltransferase.

Introduction

Higher plants produce structurally diverse and biologically active specialized metabolites, such as alkaloids, flavonoids and terpenoids (Sato 2014). Among these metabolites, the alkaloids, which are nitrogen-containing compounds, are the most biologically active and pharmaceutically important natural products. Thus, intensive characterization of alkaloids has been conducted to elucidate their chemical structures, biological activities, biosynthetic pathways, and biosynthetic enzymes and genes (Facchini 2001, Facchini and De Luca 2008, Sato 2013). Although alkaloids are usually classified based on their biosynthetic origin/pathway, benzylisoquinoline alkaloids (BIAs) are a group of alkaloids that are synthesized from tyrosine via norcooclaurine, and they are found in many plant families, including Ranunculaceae, Papaveraceae, Berberidaceae and Menispermaceae (Facchini 2001, Facchini and De Luca 2008, Ziegler and Facchini 2008). BIAs include many structurally diverse and biologically active chemicals, such as the antimalarial berberine (a protoberberine) from Coptis japonica, the narcotic analgesics morphine and codeine (morphinans) from Papaver somniferum and the antimicrobial sanguinarine (a benzophenanthridine) from California poppy (Eschscholzia californica) (Facchini 2001, Ziegler and Facchini 2008). Thus, the biosynthetic pathways and enzymes and the enzyme-coding genes have been extensively studied and characterized at the molecular level (Ziegler and Facchini 2008, Hagel and Facchini 2013, Sato and Kumagai 2013) to characterize the enzymes’ role and produce specialized metabolites in heterologous systems.

The metabolic engineering and recent reconstruction of biosynthetic pathways in microbial cells performed via synthetic biology indicated that biosynthesis is based on the characteristics...
of the enzymes. For example, during the production of reticuline from norlaudanosoline by norcoclaurine 6-O-methyltransferase (6OMT; Morishige et al. 2000), coclaurine N-methyltransferase (CNMT; Choi et al. 2002) and N-methylcoclaurine 4′-O-methyltransferase (4′OMT; Morishige et al. 2000) in Escherichia coli, it was critical for 6-O-methylation to occur prior to the subsequent reactions (Minami et al. 2008). Similarly, when stylopine was produced from reticuline by berberine bridge enzyme (BBE; Dittrich and Kutchan 1991), cheilanthifoline synthase (CYP719A5; Ikezawa et al. 2009) and stylopine synthase (CYP719A2/3; Ikezawa et al. 2007) in Pichia cells, the reactions occurred sequentially in the indicated order (Hori et al. 2016). These results indicate the importance of isolating and characterizing the biosynthetic enzymes.

In the biosynthesis of BIAs in California poppy (Eschscholzia californica), a member of the Papaveraceae family, all biosynthetic enzymes involved in the biosynthesis of reticuline to sanguinarine were identified, such as BBE (Dittrich and Kutchan 1991), CYP719A5 (Ikezawa et al. 2009), 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) and dihydrobenzophenanthridine oxidase (DBOX; Hagel et al. 2012) (Fig. 1). Moreover, these enzymes were also used to re-construct the microbial production system in yeast and Pichia cells (Fossati et al. 2014, Hori et al. 2016). However, California poppy produces many BIAs in addition to sanguinarine, including chelerythrine and macarpine, and they may be of greater medical importance (Fig. 1). Among these enzymes, chelerythrine is a well-known protein kinase C inhibitor, and macarpine shows an anti-proliferative effect on several cancer cell lines (Slaninová et al. 2007).

However, the biosynthetic enzymes for these alkaloids (such as chelerythrine and macarpine) were partly characterized at the molecular level (Fig. 1). Thus, we isolated these uncharacterized enzyme-coding genes, especially the genes from O-methyltransferases (OMTs), because they are from a well-characterized enzyme family and have conserved amino acid domains, and are crucial for the methylation process used to determine the metabolic pathways (Morishige et al. 2000, Zubiera et al. 2001, Morishige et al. 2010, Dang and Facchini 2012).

Thus far, several OMTs have been isolated and characterized in BIA biosynthesis, including 6OMT (Sató et al. 1994, Morishige et al. 2000), 4′OMT (Morishige et al. 2000), columbamine O-methyltransferase (CoOMT; Morishige et al. 2002) and scoulerine 9-O-methyltransferases (SMT in berberine biosynthesis of Coptis japonica; Takeshita et al. 1995, and SOMT in noscapine biosynthesis of Papaver somniferum; Dang and Facchini 2012). All OMTs identified in BIA biosynthesis retained the conserved S-adenosylmethionine (AdoMet)-binding domain and showed a similar sequence structure (Ziegler et al. 2001, Morishige et al. 2010, Dang and Facchini 2012). Using this conserved OMT domain as a query to search a California poppy transcriptome library (NCBI, http://www.ncbi.nlm.nih.gov; PhytoMetaSyn, www.phytometasyn.ca), several candidate OMTs were selected after further classification based on their amino acid sequence identity and expression analysis data in California poppy cell cultures with different alkaloid profiles. Full-length cDNAs of the three final candidates were isolated and expressed in E. coli to characterize their enzymological properties. The functionality of an OMT (G3) was further estimated by the in vitro reconstitution of the biosynthetic pathway using co-incubation with additional biosynthetic enzymes in BIA/benzophenanthridine alkaloid biosynthesis. Our characterization indicated that a novel OMT that is distinct from Coptis/Papaver scoulerine 9-O-methyltransferases is involved in the O-methylation of scoulerine in the biosynthesis of chelerythrine in California poppy.
Results

Isolation of the OMT candidate genes

First, using the conserved sequence of the known OMTs in California poppy (4’OMT, 6OMT and 7OMT) and C. japonica (4’OMT, 6OMT, SMT and CoOMT) (Supplementary Table S1), 118 OMT-like sequences from California poppy were obtained from the NCBI and PhytoMetaSyn expressed sequence tag (EST) databases. Based on the similarity of the AdoMet-binding domain sequence, 68 unique and uncharacterized OMT-like sequences were identified. These 68 sequences were quite different from the known OMTs in California poppy and were further allocated to 22 groups based on sequence homology of the conserved motif of OMTs, and the longest clones were used for further analysis (Supplementary Fig. S1).

Quantitative real-time PCR using group gene-specific primers (Supplementary Table S2) indicated that Groups 6, 8, 10, 12–15, 17, 20, 21 and 22 showed comparable expression in both high macarpine A5-1 and low macarpine S-38 cells, and Groups 1, 4, 5, 7, 9, 16, 18 and 19 did not show any amplification products (Fig. 2). Conversely, Groups 2, 3 and 11 showed higher expression in the high macarpine A5-1 cells (>5-fold) than in the low macarpine S-38 cells. Next, we focused on three candidates (Groups 2, 3 and 11, hereafter called G2, G3 and G11, respectively) and isolated their full-length cDNA and expressed the recombinant protein in E. coli.

The amino acid sequences from the full-length cDNA from G2, G3 and G11 showed distinct sequence similarity to the known OMTs in BIA biosynthesis (Fig. 3). G2 showed the highest sequence similarity to catechol OMT from the opium poppy (AAQ01670.1, 76% identity) and Thalictrum tuberosum (AAD29843.1, 73% identity). G3OMT showed relatively high similarity (64% and 57%, respectively) to the reticuline 7OMTs of California poppy (BAE79723.1) and opium poppy (AAQ01668.1). Conversely, G11OMT shared 35, 42 and 44% sequence identity with the flavonoid 7OMT (Hordeum vulgare; CAA54616.1), ippec OMT (Carpinopsis ippecacuanha; BAJ05383.1) and 16-hydroxytabersonine OMT (Catharanthus roseus; AB2R20103.1), respectively. Among the three OMTs, G2OMT had 39% identity with G3OMT and 30% with G11OMT, while G3OMT had 41% identity with G11OMT. The BLAST search also showed that the G3 amino acid sequence was highly similar (99% identity) to that of an uncharacterized putative O-methyltransferase (accession No. EU882970) registered by Liscombe et al. (2009).

Expression of recombinant proteins in E. coli cells

To reveal the enzymological properties of G2, G3 and G11 OMTs, the expression vectors for these OMTs were constructed and introduced into E. coli cells to produce recombinant proteins. Among the examined OMTs, only G3 showed the evident expression of the recombinant protein (Supplementary Fig. S3), and the enzyme properties of G3 were further characterized using crude and purified enzyme fractions (Supplementary Fig. S4). In the case of recombinant G11OMT, only the degraded product was detected in the insoluble fraction (Supplementary Fig. S3).

OMT activity

G3 OMT activity was determined with a set of substrates (Supplementary Fig. S5) in BIA biosynthesis with AdoMet as the methyl donor. All experiments were preliminarily performed with a crude enzyme (Supplementary Figs. S6, S7), and the results were confirmed with purified enzyme (Figs. 4, 5).

G3 showed a high similarity to the California poppy reticuline 7OMT (Ec7OMT; Fuji et al. 2007) (Fig. 3); thus, its activity was first determined with the simple benzylisoquinoline reticuline. Interestingly, G3 showed dual O-methylation activity against the hydroxy groups of reticuline at the 7 and 3’ positions and produced laudanine, codamine and laudanosoline (Fig. 4A; Supplementary Fig. S6A), whereas the recombinant Ec7OMT (Fuji et al. 2007) methylated reticuline only at position 7 and produced laudanine (Supplementary Fig. S6B).

7-O-Methylation (laudanine) and 3’-O-methylation (codamine) (3’ position of reticuline) were also confirmed by the mass fragment profiles at m/z 192 (isoquinoline moiety) and m/z 137 (benzyl moiety) of reticuline, with the increase of 14 m/z by a benzyl moiety (original m/z 137) indicating 3’-O-methylation. Full O-methylation of reticuline by G3OMT was also confirmed by the mass fragmentation profile of laudanosine (m/z 358) (Fig. 5A).

When G3 reacted with norreticuline, it also methylated norreticuline at the 7 and 3’ positions and produced norcodamine, norlaudanine and norlaudanosine (Fig. 4B). Conversely, Ec7OMT only produced norcodamine and norlaudanine from norreticuline (Supplementary Fig. S6D).

Because G3 showed unexpected reaction activities, several other substrates, including a protoberberine (scoulerine), a benzophenanthridine (10-hydroxychelerythrine) and two additional simple benzylisoquinolines (6-O-methylnorlaudanosoline and laudanosoline), were examined (Fig. 4C; Supplementary Fig. S6E, G, I, K). Among these substrates, G3 only showed O-methylation activity against scoulerine.

G3 also showed dual O-methylation activity against hydroxy groups of scoulerine at positions 9 and 2, which were identified by their mass fragmentation patterns (Figs. 4C, 5C). Specifically, products with a +14 m/z increase (i.e. m/z 342) from scoulerine by a single O-methylation were identified as 9-O-methylated scoulerine (i.e. tetrahydrocolumbamine, based on the methylation at the isoquinoline moiety) and 2-O-methylscoulerine (methylation at the benzyl moiety). G3 produced tetrahydrocolumbamine more preferentially than 2-O-methylscoulerine from scoulerine (Fig. 4C). The product with an m/z of 356 (dual O-methylation products) was determined as tetrahydropalmitamine (Fig. 5C).

Although G3 methylated and produced several products from a substrate, Ec7OMT mostly methylated at a single position (position 7) in a substrate (Supplementary Fig. S6). Ec7OMT methylated 6-O-methylnorlaudanosoline and laudanosoline to produce 6,7-O-dimethyllaudanosoline and monomethyl laudanosoline (either 6-O-methyllaudanosoline or 7-O-methyllaudanosoline; not confirmed) (Supplementary Fig. S7), respectively, whereas G3 did not methylate these...
compounds. Neither G3 nor Ec7OMT methylated 10-hydroxychelerythrine (Supplementary Fig. S6K).

Further characterization of G3 activity within a short reaction time confirmed that 7-O-methylation was preferred over 3’-O-methylation for reticuline and norreticuline and that 9-O-methylation was preferred over 2-O-methylation for scoulerine (Supplementary Fig. S8). The specificity of these reactions was obviously different from the specificity of Papaver scoulerine O-methyltransferase 1 (PsSOMT1), which also produced dual O-methylated scoulerine and reticuline but reacted sequentially (Dang and Facchini 2012).

Although G3 produced considerable amounts of tetrahydropalmatine (dual O-methylated product) from scoulerine, the production of laudanosine and norlaudanosine (dual O-methylated products) from reticuline and norreticuline, respectively, suggested that G3 would be important in the regulation of the biosynthetic pathway of BIAs of California poppy. The biological role of G3 in California poppy and the possibility of using these O-methylation activities to make new biological compounds in combination with other enzymes in benzylisoquinoline biosynthesis were examined and are described below.

Fig. 2 Quantitative RT-PCR of the expression of OMT candidate genes in S-38 (low macarpine cells) and A5-1 (high macarpine cells). Each value represents the mean ± SD of three independent experiments. nd, not detected.
Enzyme properties

First, the effects of pH, temperature, cations and certain chemicals for the OMT reaction were examined (Supplementary Figs. S9–S11). G3 showed a rather broad pH range, with optimum values from pH 6.8 to 9.6, whereas the highest activity was detected in the tricine buffer at pH 8.4 at 35°C. G3 did not require divalent cations for its activity, whereas 5 mM Ca²⁺, Co²⁺ and β-mercaptoethanol slightly inhibited the OMT activity by 7, 9 and 9%, respectively (Supplementary Fig. S11). Mg²⁺, Mn²⁺, Fe³⁺ and iodoacetamide did not inhibit G3 activity, whereas Cu²⁺, Ni²⁺ and Zn²⁺ inhibited enzyme activity by 21, 18 and 20%, respectively.

Next, we characterized the enzyme kinetics of G3 of unique dual O-methylation activity for reticuline, norreticuline and scoulerine using G3OMT, which was purified with a 6 × His tag on an Ni-NTA column (Supplementary Fig. S4). An ImageJ analysis (http://rsb.info.nih.gov/ij) indicated that G3OMT was 95.1% pure. The substrate specificities and reactivities of the purified G3 enzyme were determined at different concentrations of substrate and AdoMet under the optimized conditions (Supplementary Figs. S12, S13).

When reticuline was the substrate for G3, laudanine formation (7-O-methylation) was observed more preferentially than cadamine formation (3’-O-methylation) at all examined concentrations (Supplementary Fig. S12A), whereas PsSOMT1 produced cadamine preferentially (Dang and Facchini 2012).

When G3 reacted with norreticuline, the formation of norlaudanine (7-O-methylation) occurred preferentially over norcodamine (3’-O-methylation), whereas the reaction was slower than that for reticuline (Fig. 4B; Supplementary Fig. S12C). The formation of norcodamine was slow and not detected in the short reaction time (Supplementary Fig. S12). Interestingly, the double O-methylated product norlaudanosine (tetrahydro-papaverine; Fig. 4B) was formed from norreticuline, and G3OMT contributed to the formation of papaverine (oxidized form of tetrahydro-papaverine) from norreticuline under certain reaction conditions.

When scoulerine was reacted as the substrate at different concentrations in the presence of sufficient amounts of AdoMet, the formation of a double O-methylated product (tetrahydro-palmitamine) was clearly detected. Moreover, the formation of 9-O-methylated tetrahydrocolumbamine and 2-O-methylscoulerine was detected, especially at lower concentrations (Fig. 4C). The dose dependency curves of the
The enzyme kinetics of G3OMT for AdoMet (Supplementary Fig. S13) were determined in the presence of sufficient amounts of alkaloid substrate, and the $k_m$ values for AdoMet in the formation of laudanine and codamine from reticuline were 119 and 14.5 $\mu$M, and the $k_{cat}/k_m$ values were 1.00 and 5.51 s$^{-1}$ mM$^{-1}$, respectively. The $k_m$ value and $k_{cat}/k_m$ for AdoMet in the formation of norlaudanine were 15.7 $\mu$M and 0.64 s$^{-1}$ mM$^{-1}$, respectively, when norreticuline was used as the substrate. The $k_m$ values for AdoMet in the formation of tetrahydrocolumbamine and 2-O-scoulerine from scoulerine were 9.3 and 28.8 $\mu$M, and the $k_{cat}/k_m$ values were 1.07 and 0.35 s$^{-1}$ mM$^{-1}$, respectively.

**Estimation of the biosynthetic role of G3 in BIA biosynthesis**

The biochemical properties of G3OMT suggest its involvement in scoulerine metabolism and the biosynthesis of chelerythrine or other benzophenanthridine alkaloids. To test our hypothesis, we reacted the G3OMT reaction products with several biosynthetic enzymes in benzophenanthridine alkaloid biosynthesis: BBE (Dittrich and Kutchan 1991), chelanthifoline synthase (CYP719A5; Ikezawa et al. 2009), stylopine synthase (CYP719A2A3; Ikezawa et al. 2007), stylopine N-methyltransferase (TNMT; Liscombe and Facchini 2007) and N-methylstilboline hydroxylase (MSH; Beaudoin and Facchini 2013).

When O-methylated reticulines produced from reticuline by G3OMT were reacted with a set of biosynthetic enzymes, only laudanine was converted by BBE to 2-O-methylscoulerine (Supplementary Fig. S14A). Conversely, neither reticuline nor its O-methylated products could react with CYP719A5 and CYP719A2, thus supporting the importance of the berberine bridge ring, namely the protoberberine structure, for the CYP719A5–CYP719A2 reaction. Because California poppy cell cultures do not produce detectable levels of codamine or laudanosine, reticuline probably does not represent an in vivo substrate of G3. The mechanism underlying the inactivity of G3 against reticuline in vivo was unclear. A sieve element and cell compartment regulation of alkaloid biosynthesis (Amann et al. 1986, Ziegler and Facchini 2008) and the effect of BBE probably compete for G3OMT against reticuline.

However, when O-methylated scoulerine products were incubated, several possible intermediates of benzophenanthidine alkaloids were produced by CYP719A5, CYP719A2 or CYP719A3 (Supplementary Fig. S14B). For example, a major O-methylated scoulerine, tetrahydrocolumbamine, was converted by CYP719A3 to canadine, which was further converted to N-methylcanadine, a precursor of allocryptopine, by TNMT (Fig. 6B). Then, an N-methyl protoberberine, such as N-methylcanadine, was converted by MSH to allocryptopine. Conversely, 2-O-methylscoulerine was converted to 2-O-methylchelanthifoline by CYP719A3 only in small amounts, whereas CYP719A2 did not show any activity against O-methylated scoulerines. These results indicate the role of the functional diversification of CYP719A2 and CYP719A3 in benzophenanthridine alkaloid biosynthesis, CYP719A2 in sanguinarine biosynthesis and CYP719A3 in chelerythrine biosynthesis.

**Fig. 4** LC-MS spectra of the enzyme reactions of G3OMT with reticuline (A), norreticuline (B) and scoulerine (C). Mass ion signals were monitored with selected ion monitoring mode as described in the Materials and Methods. The red lines indicate reaction products.

substrates also showed that scoulerine was the most reactive among the three substrates (Supplementary Fig. S12E; Table 1).

The reaction kinetics for the O-methylation reaction were determined, and the substrate specificities and regio-specific reactivities were clear (Table 1; Supplementary Fig. S12B, D, F). Specifically, when reticuline was the substrate, the $K_m$ values for the formation of laudanine (7-O-methylation) and codamine (3'-O-methylation) were 393 and 187 $\mu$M, 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 7-O-methylation (norlaudanine formation) was 38.2 $\mu$M, and the $k_{cat}/k_m$ value of norlaudanine 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 $\mu$M, and the $k_{cat}/k_m$ values were 0.82 and 0.46 s$^{-1}$ mM$^{-1}$, respectively.
When O-methylated scoulerines were reacted with TNMT alone, we detected the N-methylation of scoulerine to N-methylscoulerine (m/z 342), tetrahydrocolumbamine to N-methyltetrahydro-columbamine (m/z 356) and tetrahydropalmatine to N-tetrahydropalmatine (m/z 370) (Supplementary Fig. S14B). However, the conversion of 2-O-methylscoulerine to N-methylscoulerine was not detected, and when these products were further reacted with MSH, no product was detected.

Finally, we examined the co-incubation effect of all mentioned BIA enzymes using a co-culture of *Pichia* cells expressing BIA enzymes (Hori et al. 2016). As shown in Fig. 6, the co-culture of *Pichia* cells expressing BBE, CYP719A5, CYP719A2, CYP719A3, G3OMT, TNMT and MSH produced N-methylstrepine and N-methylcanadine as the most abundant products. There was little formation of O-methylated reticuline, as mentioned above. Furthermore, an in vitro bioconversion assay indicated that BBE could actively produce more scoulerine from reticuline than the reaction with G3OMT. Moreover, the scoulerine produced was further converted by CYP719A5 and CYP719A2 to cheilanthifoline and stylopine, whereas
Table 1 Kinetic data for several scoulerine-O-methyltransferases

<table>
<thead>
<tr>
<th>Enzyme Substrate</th>
<th>Product</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsSOMT1 Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>28.5 ± 6.8</td>
<td>1.44 ± 0.27</td>
<td>50.5</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>AdoMet Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>19 ± 2.7</td>
<td>0.91 ± 0.001</td>
<td>47.89</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>Reticuline</td>
<td>Codamine</td>
<td>70.3 ± 13.7</td>
<td>0.13 ± 0.007</td>
<td>1.85</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>PsSOMT2 Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>73.3 ± 16.4</td>
<td>0.09 ± 0.013</td>
<td>1.23</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>AdoMet Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>69.62 ± 20.9</td>
<td>0.09 ± 0.04</td>
<td>1.25</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>PsSOMT3 Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>50.8 ± 13.6</td>
<td>0.06 ± 0.01</td>
<td>1.25</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>AdoMet Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>101.2 ± 29.4</td>
<td>0.07 ± 0.02</td>
<td>0.71</td>
<td>Dang and Facchini (2012)</td>
</tr>
<tr>
<td>G3 OMT Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>100.0</td>
<td>2.25</td>
<td>22.5</td>
<td>Takeshita et al. (1995)</td>
</tr>
<tr>
<td>AdoMet Scoulerine</td>
<td>Tetrahydrocolumbamine</td>
<td>170.0</td>
<td></td>
<td></td>
<td>Takeshita et al. (1995)</td>
</tr>
<tr>
<td>G3 OMT 2-O-Methylscoulerine</td>
<td>2-O-Methylscoulerine</td>
<td>24.5</td>
<td>0.02</td>
<td>0.82</td>
<td>This study</td>
</tr>
<tr>
<td>AdoMet</td>
<td>2-O-Methylscoulerine</td>
<td>21.9</td>
<td>0.01</td>
<td>0.46</td>
<td>This study</td>
</tr>
<tr>
<td>Reticuline</td>
<td>Codamine</td>
<td>9.3</td>
<td>0.01</td>
<td>1.07</td>
<td>This study</td>
</tr>
<tr>
<td>AdoMet</td>
<td>2-O-Methylscoulerine</td>
<td>28.8</td>
<td>0.01</td>
<td>0.35</td>
<td>This study</td>
</tr>
<tr>
<td>Reticuline</td>
<td>Laudanine</td>
<td>187</td>
<td>0.05</td>
<td>0.27</td>
<td>This study</td>
</tr>
<tr>
<td>AdoMet</td>
<td>Laudanine</td>
<td>393</td>
<td>0.24</td>
<td>0.61</td>
<td>This study</td>
</tr>
<tr>
<td>Norreticuline</td>
<td>Norlaudanine</td>
<td>14.5</td>
<td>0.08</td>
<td>5.5</td>
<td>This study</td>
</tr>
<tr>
<td>AdoMet</td>
<td>Norlaudanine</td>
<td>119.0</td>
<td>0.12</td>
<td>1.00</td>
<td>This study</td>
</tr>
<tr>
<td>Norreticuline</td>
<td>Norlaudamine</td>
<td>38.2</td>
<td>0.01</td>
<td>0.26</td>
<td>This study</td>
</tr>
<tr>
<td>AdoMet</td>
<td>Norlaudamine</td>
<td>15.7</td>
<td>0.01</td>
<td>0.64</td>
<td>This study</td>
</tr>
</tbody>
</table>

G3OMT could also actively produce canadine with CYP719A3. This result strongly suggests that G3OMT is the active scoulerine O-methyltransferase in vivo.

**Discussion**

In this study, we isolated the novel G3 O-methyltransferase from the California poppy, and this enzyme was active against protoberberine-type (scoulerine) and simple benzylisoquinoline-type (reticuline and norreticuline (N-methylated and N-desmethylated compounds)) alkaloids. The enzyme O-methyltransferase methylated two separate hydroxyl groups of an alkaloid (Fig. 4) despite its relatively high amino acid sequence identity (57–64%) to the known reticuline 7OMT. Thus, we denoted G3 a California poppy (E. californica) scoulerine/reticuline O-methyltransferase.

Conversely, a similar scoulerine OMT (PsSOMT1) was isolated from P. somniferum, although it functions in noscapine and papa- verine biosynthesis (Dang and Facchini 2012), which are absent in California poppy. Alternatively, California poppy produces chelerythrine-type alkaloids, which are derived from allocryptopine with one methylene dioxy ring and two methoxy groups in the benzo-phenanthridine structure. In fact, the reaction products formed from scoulerine by G3 were further converted by CYP719A3, TNMT and MSH into allocryptopine, a substrate that protopine 6-hydroxylase converts to chelerythrine (Fig. 6A, B). These results strongly suggested that the isolated G3 was the missing OMT involved in the biosynthesis of chelerythrine and related alkaloids (Fig. 1).

Interestingly, G3 was not only a scoulerine 9-O-methyltransferase, but it also O-methylated the two positions of scoulerine simultaneously. This dual activity was unique to G3 and was different from that of PsSOMT1, which showed sequential O-methylation of scoulerine to produce tetrahydropalmatine.

The phylogenetic tree analysis showed that G3 was highly similar to the reticuline 7-O-methyltransferase but distinct from the Coptis SMT and Papaver SOMT1, which preferentially worked on 9-O-methylation, and the sequence similarity among them was only 40%. Papaver SOMT2 and SOMT3 with 9-O-methylation activity for scoulerine alone showed a greater difference from G3 (sequence similarity 36%), suggesting that all scoulerine O-methylations obtained their scoulerine 9-O-methylation activity independently during evolution as proposed by Dang and Facchini (2012). Conversely, G3 showed high sequence similarity to California poppy and Papaver reticuline 7OMT, whereas PsSOMT1 also showed reticuline 7OMT activity despite belonging to a different clade, as mentioned above.

Compared with these two types of 7OMTs, G3 showed much broader regio-reactions because California poppy 7OMT only methylated reticuline and norreticuline at position 7 and scoulerine at position 2, whereas Papaver 7OMT had no activity against norreticuline and scoulerine but O-methylated position 7 of reticuline (Ounaroon et al. 2003). The current findings explain why the scoulerine O-methyltransferase gene in California poppy was not detected in a previous study that searched the Coptis SMT sequence (Takemura et al. 2010b). Despite their considerable amino acid sequence differences (Fig. 3), G3 and PsSOMT1 retained functional similarities, such as their substrate preferences (scoulerine, reticuline and norreticuline) and their dual regio-specific reactivity, which may provide clues for understanding the molecular basis of substrate/reaction specificities. However, the sequence alignment could not determine the residues involved in such multiple reactivities (Fig. 7), and their 3D structures with substrates are required as a useful basis of molecular characterization. Such characterization also provides the molecular mechanism that regulates the functional difference that allows G3 to O-
Although the physiological role of G3OMT requires reverse genetic characterization, our in vitro bioconversion assay with *Pichia* cells expressing BBE, CYP719A5, CYP719A2, CYP719A3, G3OMT, TNMT and MSH, which are involved in the early pathway in benzophenanthridine alkaloid biosynthesis, clearly suggested that these enzymes regulate the biosynthesis of canadine for chelerythrine. The *k*_cat/*K*_m* values of G3OMT for scoulerine were not high; thus, a high affinity (small *K*_m) for scoulerine would be favorable for the reactions.

Each O-methyltransferase has several conserved motifs of catalytic domains. Motif I (nine amino acids), a glycine-rich sequence, motif II (eight amino acids) and motif III (10 amino acids) are easily identified in methyltransferases (Fig. 7; Kagan and Clarke 1994, O’Gara et al. 1995, Struck et al. 2012). Glycine residues in motif I form a tight loop responsible for positioning AdoMet in the binding pocket. Conversely, rich aromatic residues in motif II could be bound to positively charged compounds, such as cations, by interactions with electrons in the aromatic ring. Motif I, II and III are better characterized than other motifs in methyltransferases (Joshi and Chiang 1998).

Recently, the crystal structure of an OMT involved in BIA biosynthesis, *Thalictrum flavum* 6OMT (Tf6OMT), was determined with SAH (S-adenosyl-1-homocysteine; an AdoMet analog) and its substrate norlaudanosoline (Robin et al. 2016). However, the sequence similarities among Tf6OMT, scoulerine OMTs and reticuline 7OMTs were not high, with Tf6OMT showing 38, 31, 39, 59, 39 and 39% identity to G3, PsSOMT1, PsSOMT2, PsSOMT3, CjSMT, Ec7OMT and Ps7OMT, respectively. This result only confirmed the conservation of AdoMet-binding motifs and the general conservation of its secondary structure. The SAH-binding residues (G195 in motif I, D218 in motif II, D228 in motif III, and K252 in motif IV) found in Tf6OMT were also found in G3 at G206, D229, D249 and K263. Moreover, the catalytic H256 and D227 in Tf6OMT were found in G3 as H267 and D228, respectively.

Conversely, the substrate-binding residues of Tf6OMT were not conserved in scoulerine OMT or 7OMTs. Specifically, D169, C253 and D236 of Tf6OMT were found in G3 as H267 and D268, respectively. Black peaks represent the starting materials for bioconversion, i.e. reticuline or canadine, and the red peaks indicate the newly formed products via the enzyme addition. BBE, berberine bridge enzyme; CYP719A2, styloïne synthase, CYP719A3, styloïne/canadine synthase; CYP719A5, cheilanthifoline synthase; MSH, N-methylstylopine 14-hydroxylase; and TNMT, tetrahydroprotoberberine N-methyltransferase.

![Fig. 6](https://example.com/fig6.png) Conversion of reticuline as a substrate with transgenic *Pichia* cells expressing BIA biosynthetic enzyme analyzed with an LC-MS 8030 (Shimadzu). The substrate was reacted with BIA biosynthetic enzymes expressed in *Pichia pastoris* G5-115 cells with the pPIC3.5.K expression vector. The products were identified using authentic samples and/or fragmentation patterns of products: m/z 326, cheilanthifoline; m/z 328, scoulerine; m/z 330, reticuline; m/z 338, N-methylstylopine; m/z 340 a, N-methylcheilanthifoline; m/z 340 b, 2-O-methylcheilanthifoline; m/z 340 c, canadine; m/z 342 a, N-methylscoulerine; m/z 342 b, tetrahydrocolumbianine; m/z 342 c, 2-O-methylscoulerine; m/z 344, codamine; m/z 354 a, N-methyl-2-O-methylcheilanthifoline; m/z 354 b, N-methylcanadine; m/z 370 a, N-methyltetrahydropalmatine; m/z 370 b, allocryptopine, respectively. As mentioned above, scoulerine OMTs and reticuline 7OMTs showed considerable sequence diversities and broad reaction specificities. This diversity of OMTs would be useful for analyzing the structural importance of enzyme activities, the molecular evolution of OMTs and the method by which they acquired functional similarity and uniqueness with their diversified structures.

Among the OMTs involved in BIA biosynthesis in California poppy, G3OMT showed a wider regio-specific reactivity (Table 1) as evidenced in the O-methylation activity of protoberberine at the 9 and 2 positions and simple benzylisouquinoline at the 7 and 3 positions. However, reticuline 7OMT activity was only detected when the high accumulation of the substrate reticuline was achieved by RNA interference (RNAi) of BBE in California poppy (Fujii et al. 2007). Similarly, G3 activity other than scoulerine 9OMT might not be evident under normal physiological conditions in which other biosynthetic enzyme functions and the accumulation of substrate were not induced.

methylate both positions 9 and 2 of scoulerine simultaneously while PsSOMT1 only methylates scoulerine at position 9 and then catalyzes the second methylation at position 2 (Dang and Facchini 2012).
Moreover, the expression of G3 was low in the S-38 cells with high 10-hydroxychelerythrine and high in the A5-1 cells with high macarpine (Fig. 2; Supplementary Fig. S2). Although this result suggests that G3 is of little importance in chelerythrine biosynthesis, S-38 cells are transformants that present the high expression of *Coptis* SMT. Thus, we expected that such expression of *Coptis* SMT reduced the expression of endogeneous G3 for chelerythrine biosynthesis. This unexpected interference of gene expression for similar enzyme functions would be an interesting subject for future investigations.

**Fig. 7** Sequence alignment G3 (Ec scoulerine/reticuline OMT) and known scoulerine or reticuline OMTs. The sequences were aligned using BioEdit Sequence Alignment Editor. PsSOMT1, scoulerine 9-OMT-1 (*P. somniferum*, AFB74611.1); PsSOMT2, scoulerine 9-OMT-2 (*P. somniferum*, AFB74612.1); PsSOMT3, scoulerine 9-OMT-3 (*P. somniferum*, AFB74613.1); CjSMT, scoulerine 9-OMT (*C. japonica*, BAA06192.1); Ec7OMT, reticuline 7-OMT (*E. californica*, BAE79723.1); Ps7OMT, reticuline 7-OMT (*P. somniferum*, AAQ01668.1); and Tf6OMT, norcoclaurine-6-OMT (*T. flavum*, AY610507). The green highlights show the conserved residues for SAH binding, and the red highlights show the conserved histidine and aspartate residues in the catalytic domain.
When we reconstruct the biosynthetic pathways using isolated biosynthetic enzyme-coding genes, new biosynthetic pathways are constructed with any enzyme-coding genes, including those from non-plant origins, and with all reactions. The broad reactivity of G3 would be useful for designing the biosynthetic pathways to re-construct alkaloid biosynthesis in microbes (Minami et al. 2008, Hori et al. 2016). Papaverone biosynthesis is interesting because G3 could convert norreticuline to norlaudanosoline (tetrahydropapaverine) in vitro. G3 may open up a new field of enzymological conversion of BIs (Supplementary Fig. S15).

Expression of recombinant protein in E. coli

Expression vectors containing the full-length OMT cDNA were introduced into E. coli BL21 (DE3), and transgenic E. coli cells were grown in LB medium at 200 r.p.m. and 37°C. After the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to OD600 0.6–0.8, the E. coli cells were further incubated at 16°C for 24 h. The E. coli cells were harvested by centrifugation, and recombinant proteins were extracted by sonication in extraction buffers containing 100 mM potassium phosphate (pH 8.0), 10% glycerol, 5 mM β-mercaptoethanol and 5 mM sodium EDTA. Homogenates were centrifuged at 13,000 r.p.m., and the supernatants were desalted with PD10 (GE Healthcare) and used as crude enzymes for the enzyme assay and SDS–PAGE analysis (Supplementary Fig. S3). EcOMT was expressed as described previously (Fuji et al. 2007).

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 (~50 μg of crude protein or 5 μg of purified protein) at 35°C in triplicate. Preliminary OMT assays were done with 1 h reactions of crude enzymes (Supplementary Fig. S6). Assays for pH optimum, temperature optimum and effects of chemicals were done with 15 min reactions of crude enzymes with 100 μM scoulerine (Supplementary Figs. S9–S11). For pH optimum assay, 100 mM buffer with HEPES (Djoindo), tricine (Djoindo), N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS, Djoindo), N-cyclohexyl-2-aminoethanesulfonic acid (CHES), and K2HPO4 and KH2PO4 (Nacalai tesque) were used. OMT assays with the purified enzymes were done for 1 h (Fig. 4) or 20 min (Supplementary Figs. S12, S13).

After the enzymatic reactions were stopped by the addition of an equal volume of methanol containing 4% trichloroacetic acid, the proteins were sedimented via centrifugation. The reaction products were analyzed using an LC-MS 2020 (Shimadzu) with the following system: a TSKgel ODS-80 TSK column (4.6 mm i.d. x 250 mm, 5 μm, TOSOH), 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⁻¹ at 40°C. In the case of the substrate 10-hydroxychelerythrine, 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 1.5 kV (positive ion mode), and the UV spectrum at 190–600 nm measured by a photodiode array detector. MS fragment spectra of alkaldoids were detected by the LC-MS 8030 (Shimadzu) system; ESI–MS with product ion scan mode, m/z 50.00–400.00, collision energy at ~35 V.

Characterization of enzymological properties using the purified recombinant G3 OMT

To determine the enzymatic properties of the purified G3OMT, a His-tagged G3OMT expression vector was constructed, and the proteins expressed in E. coli were purified using an Ni-NTA column. Briefly, six histidine tags were added to the 3′ region of G3OMT and cloned to the NdeI and XhoI restriction sites at the 5′ and 3′ ends in PET 22(b) (Novagen). Production of a recombinant protein in E. coli BL21 (DE3) harboring expression vectors was induced by IPTG for 24 h at 16°C and extracted in 20 ml of buffer by sonication. After the precipitation of cell debris by centrifugation at 13,000 r.p.m. for 15 min, the supernatant was applied to 10 ml of Ni-NTA resin (Roche) at a flow rate of 0.5 ml min⁻¹. After washing with a sufficient volume of buffer A (50 mM sodium phosphate buffer, pH 8.0, containing 400 mM NaCl), His-tagged G3OMT was eluted with buffer A containing 75 and 100 mM imidazole. The eluted purified fractions were

Materials and Methods

Plant material

Cultured California poppy (Eschscholzia californica) cells (low macarpine 5–38 line overexpressing the G3OMT gene (Takemura et al. 2010b) and high macarpine AS-1 line overexpressing EcCYTP191AS gene (Takemura et al. 2010a)) were established in our laboratory and subcultured every 3 weeks in Linsmaier–Skog medium containing 10 μM naphthaleine acetic acid and 1 μM benzylademenine with 3% sucrose. Two-day-old cultured cells were harvested and used for mRNA extraction using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was prepared from total RNA using a Prime Script RT reagent Kit (TAKARA BIO INC.) based on the manufacturer’s instructions as described previously (Yamada and Sato 2016).

Chemicals

(S)-Laudanosoline was purchased from Aldrich, and (R,S)-reticuline, (R,S)-6-O-methylnorlaudanosoline, (R,S)-norreticuline and (S)-scoulerine were a kind gift from Mitsui Petrochemical. The compound 10-hydroxychelerythrine was purified from the California poppy S-38 cells using Combiflash (Teledyne Isco), and (S)-tetrahydrocolumbamine was enzymatically prepared from (S)-scoulerine (Ikezawa et al. 2003). Norlaudanosine was prepared from papaverine via a chemical reduction with sodium borohydride.

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

OMT candidate genes were screened from the cDNA databases of the NCBI (http://www.ncbi.nlm.nih.gov) and PhytomeTasyn (www.phytomeTasyn.ca) using a known sequence of OMTs of California poppy (4’OMT, 6OMT and 7OMT) and C. japonica (4’OMT, 6OMT, SMT and CoOMT) found in BIA biosynthesis (Supplementary Table S1). The 118 OMT-like sequences were grouped based on the similarity of the AdoMet-binding domain sequence, and 68 unique and uncharacterized OMT-like sequences were identified. The phylogenetic tree was created with MEGA 6.0 software (Tamura et al. 2013). According to the sequence homology in a phylogenetic tree, the longest 22 representative gene candidates were selected for the expression profile analysis. Because the isolated amino acid sequences were not full length, the sequences between the conserved motif I and motif III of the OMTs were used for the phylogenetic analysis (approximately 110 amino acids) in Supplementary Fig. S1.

The expression of 22 representative genes was examined in the following two cell lines of California poppy with different alkaloid profiles to isolate each specific OMT (Table S2): a high macarpine-producing AS-1 cell line (Takemura et al. 2010a) and a high macarpine-producing line, which overexpresses the rate-limiting CYTP191AS gene, and a low macarpine- but high 10-hydroxychelerythrine-producing S-38 cell line, which overexpresses Capsis SMT to channel the metabolic flow from the macarpine type to chelerythrine type alkaloids.

cDNAs of the S-38 and AS-1 cells were synthesized from total mRNA of 2-day-old cultured cells. A set of primers for the entire group (Supplementary Table S1) was designed for the quantitative RT-PCR to produce approximately 80–170 bp fragments. Actin was used as the housekeeping gene to normalize the expression of the OMT candidate genes.
desalted on a PD-10 column, concentrated by Amicon Ultra-15 (Sigma) and stored at 1.2 mg ml⁻¹ in a solution of 100 mM potassium phosphate buffer (pH 7.2) with 40% glycerol until use (Supplementary Fig. S4). All operations were performed at 4 °C. The protein concentration was determined according to the Bradford method with bovine serum albumin as the standard.

**Reconstruction of biosynthetic pathway with recombinant proteins**

To evaluate the physiological role of the biosynthetic enzyme G3 and its use in biotechnological applications to produce more novel compounds, we reacted the O-methylation products of reticuline and scoulerine produced by G3 with several biosynthetic enzymes in BIA pathways. For substrate preparation (O-methylated reticulines and scoulerines), 200 μM reticuline and scoulerine were reacted with 100 μg of G3 crude enzyme in 100 mM tricine buffer (pH 8.4) containing 10% glycerol, 5 mM β-mercaptoethanol, 5 mM sodium EDTA and 0.5 mM AdoMet for 1 h. After enzyme inactivation by methanol and centrifugation, the supernatants containing the reaction products were first recovered using a Sep-Pak™ column and then eluted with methanol. The supernatants were then evaporated to dryness and resolved in 50 μl of dimethylsulfoxide (DMSO) for the reaction with Pichia cells harboring one of the following enzyme-coding genes: BBE, CYP719A5, CYP719A2, CYP719A3, TNMT and MSH, of California poppy in pPIC3.5 K (Hori et al. 2016). Pichia cells grown in 1 ml of YPD (yeast extract, peptone, dextrose) medium for 24 h at 30 °C were suspended in the BMMY medium, and gene expression was induced by methanol. After 24 h induction, 15 μl of substrate solution was added and incubated for 48 h, and 0.5% methanol was added every 24 h.

For incubation with a mixture of Pichia cells expressing BIA biosynthetic enzymes (Fig. 6A, B), 200 μM substrate (reticuline or canadine) was added and incubated for 96 h with the addition of 0.5% methanol every 24 h. Metabolites were extracted from Pichia cells in methanol containing 0.01 N HCl via sonication for 60 min and then analyzed with an LC-MS/MS 8030 (Shimadzu).

**Supplementary data**

Supplementary data are available at PCP online.

**Funding**

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) [Grant-in-Aid for Scientific Research (S), 26221201 to F.S.].

**Acknowledgments**

We thank Mitsui Petrochemical for their gifts of alkaloids. A.P., K.H. and F.S. designed the research; A.P. performed the experiments; A.P., K.H., Y.Y. and F.S. analyzed the data; A.P. and F.S. wrote the manuscript, and all authors reviewed the manuscript.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


