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REVIEW

Plant Flavonoid *O*-Methyltransferases: Substrate Specificity and Application

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Abstract Flavonoids consist of a large family of compounds, which has been estimated to be more than 10,000 compounds. The structural diversity of these compounds comes from different modification reactions. The *O*-methylation reaction is one of the most important modification reactions of flavonoids and the resulting *O*-methylated flavonoids have been shown to display new biological activities. The regioselective and substrate specific *O*-methylation is mediated by *O*-methyltranferases (OMTs). To date, 30 flavonoid OMTs (FOMTs) have been biochemically characterized from various plants. FOMTs utilize common reaction mechanisms to transfer a methyl group to the hydroxyl group of the flavonoid. Phylogenetic tree analysis along with biochemical characterization of FOMTs provides clues about their substrate specificity and regioselectivity. FOMTs can be used for the production of *O*-methylated flavonoids that have a particular biological activity.

Keywords Flavonoid · *O*-Methylation · *O*-Methyltransferase · Regioselectivity · Substrate specificity

Introduction

Flavonoids are a large group of phytochemicals which has been estimated to include more than 10,000 compounds (Tahara 2007). These compounds are derived from the primary metabolism of phenylalanine. The entry point of the flavonoid biosynthesis is cinnamic acid, which is produced by first reaction product of phenylalanine with phenylalanine lyase. Cinnamic acid, which is also called phenylpropanoid, serves as the substrate for lignin biosynthesis and flavonoid biosynthesis. Chalcone synthase, which condenses one molecule of 4-courmaryol-CoA and three molecules of malonyl-CoA, is the first enzyme used in flavonoid biosynthesis (Winkel-Shirley 2001a). Following this reaction, several modification reactions including hydroxylation, methylation, glycosylation, and malonylation occur to produce individual flavonoids.

Flavonoids are classified into flavane, flavanone, flavonol, flavone, isoflavone, isoflavanone, and anthocyanin depending on the carbonyl group on carbon 2, the double bond between carbon 2 and 3, the presence of a hydroxyl group on carbon 3, and the location of the B ring (Fig. 1; Forkmann and Heller 1999). During the biosynthesis of flavonoids, several hydroxyl groups are added and the most predominant positions where they are added include the 3, 5, 6, 7, 3′, 4′, and 5′ positions. The position and the number of hydroxyl groups vary among different flavonoids. The hydroxyl groups of flavonoids are prone to undergo methylation or glycosylation. In particular, *O*-methylation of hydroxyl groups in flavonoids reduces their reactivity and increases their antimicrobial activity (Ibrahim et al. 1998). In nature, various *O*-methylated flavonoids have been found, most of which contain distinct biological activities (see below).

O-Methylation of flavonoids is carried out by *O*-methyltransferase (OMT), which uses *S*-adenosylmethionine (SAM) as a methyl group donor and the flavonoid as a methyl group acceptor. OMTs are categorized into two classes; caffeoyl-CoA OMT (CCoAOMT) type and caffeic acid OMT (COMT) type (Joshi and Chiang 1998). Classification of OMTs is based on the molecular weight and

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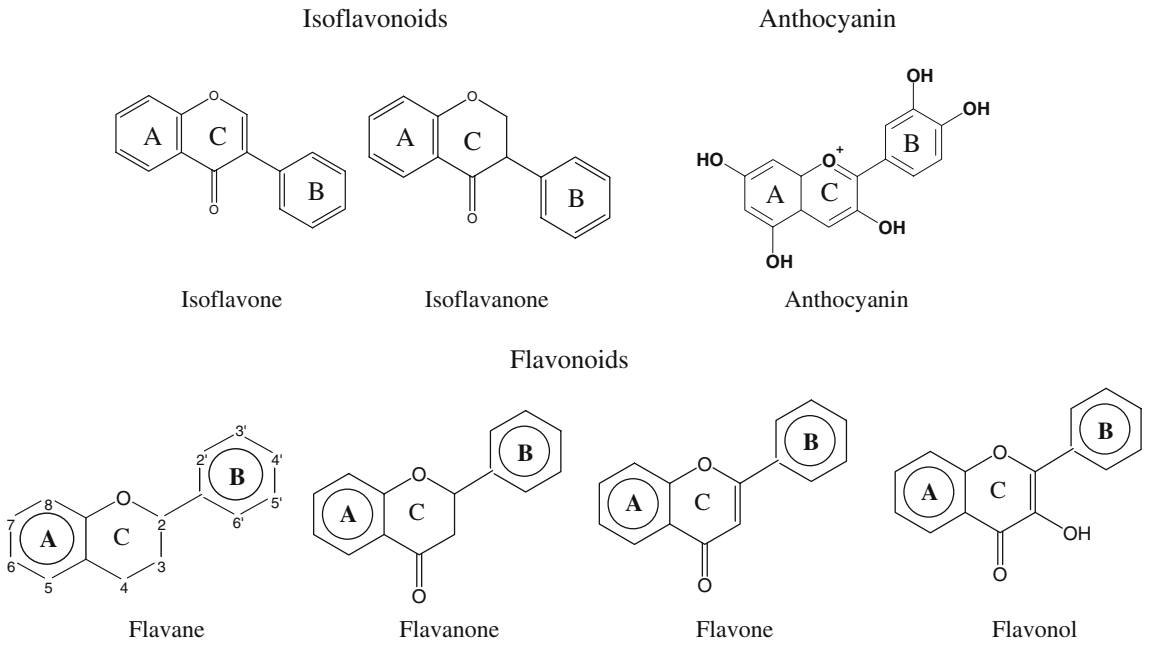

Fig. 1 Structure of flavonoids

bivalent ion dependency. The CCoAOMT type is involved in the biosynthesis of monolignols, its molecular weight ranges from 26 to 30 kDa and its activity is dependent on the presence of bivalent ions. Subgroups of the CCoAOMT type also use flavonoid as well as phenylpropanoids (Ibdah et al. 2003; Lee et al. 2008; Kim et al. 2010a). FOMTs belong to the COMT type whose molecular weight ranges from 40 to 43 kDa. In addition, the activity of FOMTs is independent of biovalent ion. In this review, we will focus on only COMT-type FOMTs.

Substrate Specificity and Regioselectivity of Flavonoid OMTs

Thirty FOMTs have been characterized biochemically so far but the gene for 3-OMT has not yet been cloned (Table 1). Among the several hydroxyl groups found in flavonoids, the 5-hydroxyl group cannot be easily methylated or glycosylated because the 5-hydroxyl group can form a hydrogen bond with the carbonyl group at carbon 3. However, naturally 5-hydroxyl group substituent has been found (Vogt et al. 1988; Ngadjui et al. 1995). The phylogenetic tree of these FOMTs was constructed. Except for F1-OMT, all other FOMTs were clustered into two groups. The first group (group I) uses flavonoid as a substrate and most of the second group (group II) use isoflavonoids as a substrate (Fig. 2). Within group I, the largest class consists of 3′ or 3′ and 5′ FOMT and group II primarily consists of 7 or 4′ FOMT. The regioselectivity of each FOMT is the basis of the phylogenetic tree.

As shown in Table 1, the most common FOMT are the flavonoid 3′-OMT. From the biosynthesis of the flavonoid via phenylalanine lyase, naringenine, which has three hydroxyl groups at 5, 7, and 4′, is the first synthesized flavonoid. The 3′-hydroxylation of flavanone or flavonol is carried out by flavonoid 3′-hydroxylase, which is a member of the cytochrome P450 (Winkel-Shirley 2001b). Flavonoids containing 3′ hydroxyl groups are represented by luteolin, myricetin, qercetin, and tricetin. Luteolin and quercetin have two hydroxyl groups in the ring B (3′ and 4′ hydroxyl groups). Reaction of either ROMT9, TaOMT2, HvOMT1, or ZmOMT1 with luteolin or quercetin produces a 3′-*O*-methylated product. However, tricetin or myricetin, both of which contain three hydroxyl groups (3′, 4′, and 5′) in the B ring, produces a 3′, 5′-*O*-dimethylated product (Zhou et al. 2006, 2008; Kim et al. 2006c; Hong et al. 2009). This occurs because the 3′ and 5′ hydroxyl groups in myricetin and tricetin are chemically equivalent. However, in this reaction, a monomethylated product is rarely observed. It seems that the monomethylated product is rotated quickly for the second methylation reaction without leaving the active site (Hong et al. 2009). The glucosides of 3′, 5′-*O*-dimethyltricetin, tricin, is commonly found in the bran of several cereals and contain various biological activity including inhibiting breast tumor cells and colon cancer cells (Hudson et al. 2000; Cai et al. 2004). The 3′-OMTs found in outside of cereal do not contain 3′,5′-*O*-dimethyltransferase activity. For example, VanOMT-3 from *Vanilla planifolia* converts myricetin only into 3′-*O*-methylmyricetin (laricitrin; Li et al., 2006). CrOMT2 from *Catharanthus roseus* also

Table 1 Biochemically characterized flavonoid OMTs

Name	Origin	Substrate specificity or regioselectivity	Accession number	Reference
AtOMT1	*Arabidopsis thaliana*	Flavonol 3′-OMT	U70424	Muzac et al. 2000
CrOMT2	*Catharanthus roseus*	3′5′-*O*-dimethyltransferase of myricetin	AY127568	Cacace et al. 2003
CrOMT6	*Catharanthus roseus*	Homoeriodictyol 4′-*O*-methyltransferase	AY343490	Schröder et al. 2004
F1-OMT	*Hordeum vulgare*	Flavone 7-OMT	X77567	Christensen et al. 1998
GeHI4′OMT	*Glycyrrhiza echinata*	2,7,4′-trihydroxyisoflavanone 4′-O-methyltransferase	AB091684	Akashi et al. 2003
HvOMT1	*Hordeum vulgare*	Flavone 3′-*O*-methyltransferase or Flavone 3′, 5′-*O*-dimethyltransferase	BarleyBase 002N0[a]	Zhou et al. 2008
IOMT	*Medicago sativa*	Isoflavone 4′-OMT	U97125	He et al. 1998
LjHI4′OMT	*Lotus japonicus*	2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase	AB091686	Akashi et al. 2003
MpOMT1A	*Mentha x piperita*	Flavonol 7-OMT	AY337457	Willits et al. 2004
MpOMT1B	*Mentha x piperita*	Flavonol 7-OMT	AY337458	Willits et al. 2004
MpOMT2	*Mentha x piperita*	Flavonol 8-OMT	AY337459	Willits et al. (2004)
MpOMT3	*Mentha x piperita*	Flavonol 3′-OMT	AY337460	Willits et al. 2004
MpOMT4	*Mentha x piperita*	Flavonol 4′-OMT	AY337461	Willits et al. 2004
MtIOMT1	*Medicago truncatula*	Glycitein 7-*O*-methyltransferase	AY942159.1	Deavours et al. 2006
MtIOMT2	*Medicago truncatula*	Daidzein 7-*O*-methyltransferase	DQ419910	Deavours et al. 2006
MtIOMT3	*Medicago truncatula*	6,7,4′-trihydroxyisoflavone 7-*O*-methyltransferase	DQ419911.1	Deavours et al. 2006
MtIOMT4	*Medicago truncatula*	Coumestrol 3,9-dimethyltransferase	DQ419912	Deavours et al. 2006
MtIOMT5	*Medicago truncatula*	2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferase	AY942158.1	Deavours et al. 2006
MtIOMT6	*Medicago truncatula*	Dihydrodaidzein 4′, 7-di-*O*-methyltransferase	DQ419913.1	Deavours et al. 2006
MtIOMT7	*Medicago truncatula*	Naringenin 7-*O*-methyltransferase	DQ419914.1	Deavours et al. 2006
OMT1	*Chrysosplenium americanum*	Luteolin 3′-OMT	U16793[b]	Gauthier et al. 1998
OMT2	*Chrysosplenium americanum*	Luteolin 3′-OMT	U16793[b]	Gauthier et al. 1998
3-OMT	*Serratula tinctoria*	Flavonol 3-OMT	NA[c]	Huang et al. 2004
pFOMT3′	*Chrysosplenum americanum*	3′/5′-O-methylation of partially methylated flavonols	U16794	Gauthier et al. 1996
POMT7	*Populus deltoids*	Flavones or flavonol 7-*O*-methyltransferae	TC29789[d]	Kim et al. 2006b
ROMT9	*Oryza sativa*	Flavone 3′-*O*-methyltransferase or Flavone 3′, 5′-*O*-dimethyltransferase	29893141	Kim et al. 2006c
SaOMT2	*Streptomyces avemilitils*	Flavonoid and isoflavonoid 7-OMT	1213742	Kim et al. 2006a
SOMT2	*Glycine max*	Flavanone 4′-OMT	TC178411[d]	Kim et al. 2005b
TaOMT2	*Triticum aaestivum*	3′,5′-*O*-dimehtyltransferase		Zhou et al. 2006
Van OMT-3	*Vanilla plaifolia*	Myricetin 3′-OMT (no dimethylation product, no activity on querecetin)	DQ400400	Li et al. 2006
ZmOMT1	*Zea mays*	Flavone 3′-*O*-methyltransferase or Flavone 3′, 5′-*O*-dimethyltransferase	BarleyBase 0040C07	Zhou et al. 2008

[a] BarleyBase (http://www.plexdb.org) accession number

[b] Only three amino acids out of 343 amino acids are different in OMT1 and OMT2

[c] *NA* not available because the corresponding gene has not been cloned

[d] TIGR (The Institute of Genome Research) accession number

produces 3′,5′-*O*-dimethylmyricetin (Cacace et al. 2003). However, it displays low activity toward quercetin. pFOMT3′ from *Chrysosplenum americanum* uses only 3′-*O*-methylated flavonoid to produce 3′,5′-*O*-dimehtyl flavonoid but does not use unmethylated flavonoid (Gauthier et al. 1996).

7-OMTs have been shown to display substrate specificity. Ten FOMTs have been reported to display 7-OMT

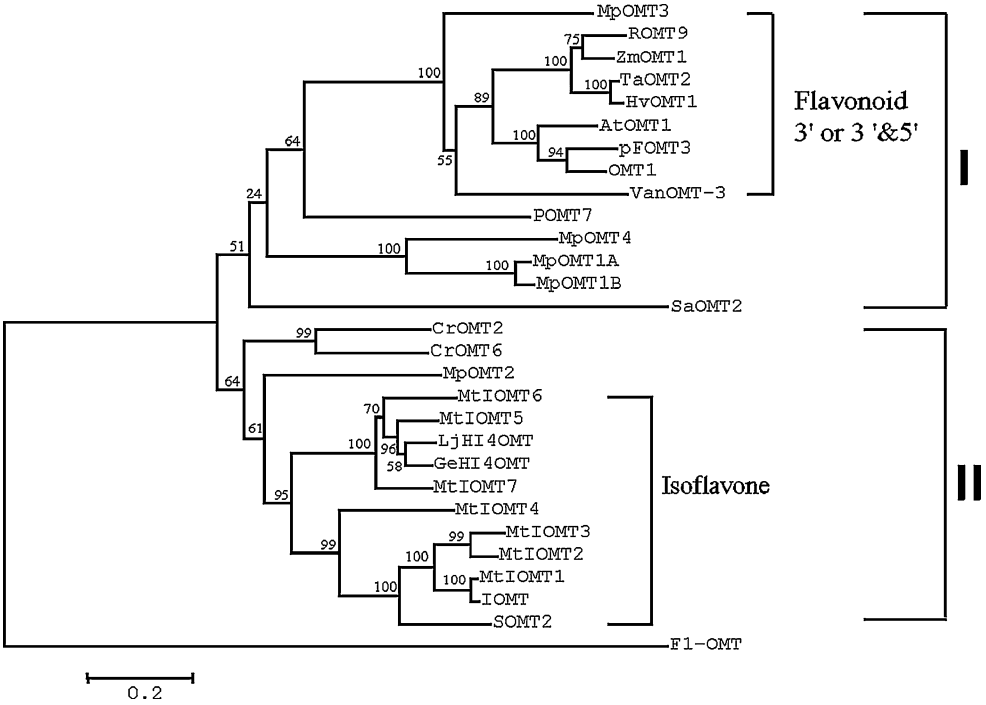

Fig. 2 Phylogenetic tree of flavonoid OMTs. Bootstrap values, which indicate the percentage values for obtaining this particular branching in 1,000 repetitions of the analysis are represented as numbers at the forks. Mega 4 was used for generating this tree

activity although some of these FOMTs also transfer methyl groups to both 7 and 4′-hydroxyl groups. 7-OMT can be divided into two groups depending on the substrate; flavonoid or isoflavonoid. Most of them are exclusive activity, where 7-OMTs that use flavonoids as a substrate do not use isoflavonoids as a substrate and vice versa. But SaOMT2 from *Streptomyces avemilitis* uses not only flavonoids but also isoflavonoids as a substrate with almost the same preference (Kim et al. 2006a). It seems that OMTs from microorganisms contain more flexibility than those from plants in regards to substrate specificity (Kopycki et al. 2008; Lee et al. 2006). Most 7-OMTs from *Medicago truncatula* use isoflavonoids although MtIOMT7 uses flavanone (naringenin) better than isoflavanone (Deavours et al. 2006). MtIOMT1, MtIOMT2, and MtIOMT3 that use isoflavone as the most preferable substrate over isoflavane are clustered together in the phylogenetic tree and show a high sequence identity (more than 79% amino acid sequence identity). These three enzymes transfer a methyl group onto the 7-hydroxyl group of the isoflavone substrate. Likewise, MtIOMT5 and MtIOMT6 use isoflavane as the most favorable substrate and have more than 75% amino acid sequence identity. MtIOMT7 whose most favorable substrate is flavanone (naringenin), shows more amino acid similarity to MtIOMT5 and MtIOMT6 than MtOMT1-3. Thus, it seems that the double bond between carbon 2 and 3 is a more important factor in discriminating substrate specificity than the location of the B ring in at least these MtIOMTs.

Among the MtIOMTs that use the same group of isoflavonoid as a substrate, each enzyme can distinguish subtle differences in the structure of the substrate. These three isoflavones, diadzein (7,4′-dihydroxyisoflavone), glycitein (7,4′-dihydroxy-6-methoxyisoflavone), and 6,7,4′-trihydroxyisoflavone have very similar structures where the only difference is the number of hydroxyl groups (6,7,4′-trihydroxyisoflavone has one more hydroxyl group compared with diadzein) and the presence of a methoxy group (glycitein has methoxy group compared with diadzein). However, MtIOMTs can distinguish these differences. It would be interesting to determine which amino acids in these MtIOMTs recognize these subtle differences in the substrate structure.

The 7-OMTs that use flavonoids as a substrate do not show high amino acid sequence similarity to one another. F1-OMT, MpOMT1s, and POMT7 prefer flavones or flavonols over flavanones (Christensen et al. 1998; Willits et al. 2004; Kim et al. 2006b). However, SaOMT2 show compatible activity toward flavanones and isoflavones as well as flavones and flavonols (Kim et al. 2006a). POMT7 regioselectively methylates the 7-hyroxyl group. However, it alters its regioselectivity from the 7 to 4′-hyroxyl group when 3′-*O*-methylated flavones are used as a substrate (Kim et al. 2008). The change in regioselectivity of OMTs as observed in POMT7 might contribute to the production of more methylated flavonoids in nature.

The 4′-OMT like the 7-OMT can be divided into two groups depending on the location of the B ring; flavonoids and isoflavonoids. The 4′-OMTs that use isoflavonoids as a

substrate include GeHI4OMT, LjHI4OMT, IOMT, MtIOMT5, and MtIOMT6 (He et al. 1998; Akashi et al. 2003; Willits et al. 2004). Among the 4′-OMTs, all but IOMT prefer isoflavanes as substrates. MtIOMT6 methylates not only the 4′-hydroxyl group but also the 7-hyroxyl group of isoflavanes. CrOMT6, SOMT2 and MpOMT4 use flavonoids as a substrate (Kim et al. 2005a, b; Schröder et al. 2004; Willits et al. 2004). CrOMT6 and MpOMT4 have a higher activity toward monomethylated flavonoid than nonmethylated flavonoid. CrOMT6 exclusively methylates homoeriodictyol (3′-*O*-methyleriodicyol) and the most favorable substrate of MpOMT4 is isorhamnetin (3′-*O*-methylquercetin).

Flavonol 3-OMT was partially purified from *Serratula tinctoria* (Huang et al. 2004) but the corresponding gene has not yet been cloned. This is the only flavonol 3-OMT that has been characterized. By screening an error-prone polymerase chain reaction library of POMT7, flavones a 3,7-*O*-dimethyltransferase that uses flavones as a substrate was recently generated (Joe et al. 2010). An 8-OMT was also cloned from Peppermint (Willits et al. 2004). Since most flavonoids found in nature contain 3, 5, 7, 3′, 4′, or/and 5′ hydroxyl groups, this 8-OMT was unusual. A hydroxylase specific to carbon 8 of flavonoids has not yet been found.

The Mechanism of Flavonoid *O*-methylation

IOMT was the first FOMT whose structure was determined using X-ray crystallography (Zubieta et al. 2001; Liu et al. 2006). The overall structure of IOMT as well as those of other OMTs belonging to COMT shows that the C-terminal is responsible for SAM and flavonoid binding and the N-terminal part is involved in dimmerization. The catalytic mechanism of FOMT was elucidated based on the structure of the IOMT. In the substrate-binding pocket of IOMT, His 258 is positioned close to the methyl group acceptor, 7-hydroxyl group of daidzein, and serves as a base for deprotonation. The methyl group of the cosubstrate, SAM is also positioned close to the deprotonated hydroxyl group of daidzein where the subsequent methylation reaction occurs. Thus, this histidine residue is important for its catalysis activity. Alignments of 29 FOMTs reveal that the histine residue is conserved across in FOMTs except F1-OMT, which contains an arginine at this position (Fig. 3).

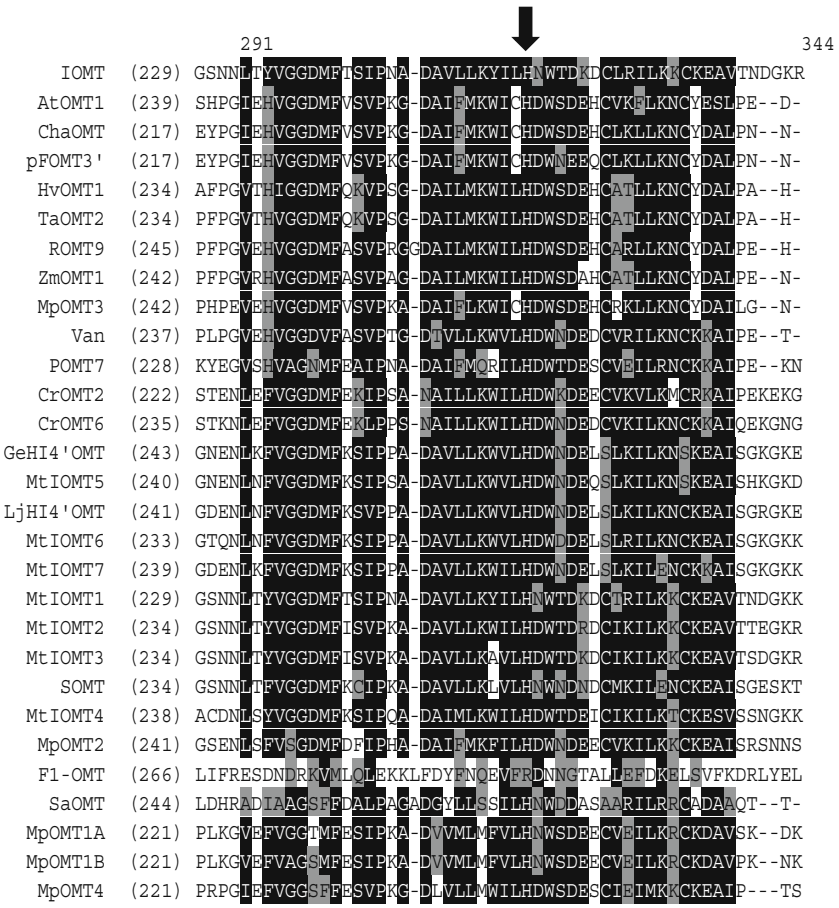

Fig. 3 Alignment of amino acids around the histidine residue, which serves as a base for the *O*-methylation reaction in FOMTs. *Arrow* indicates the conserved histidine

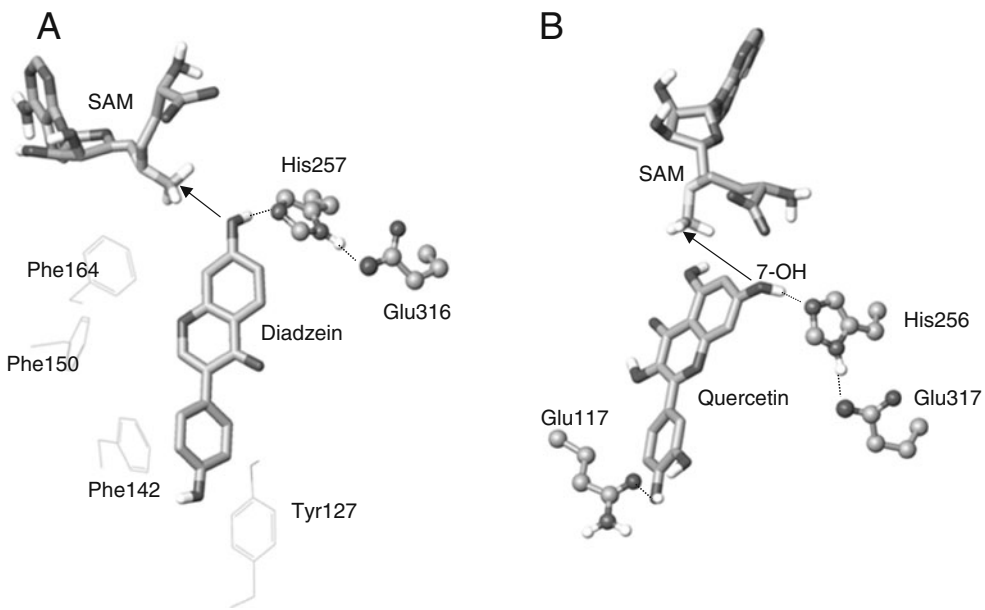

Fig. 4 The substrate binding pocket of IOMT (**a**) and POMT7 (**b**)

As mentioned above, divalent metal ions are not needed for COMT activity. In the case of CCoAOMT-type OMTs, the bivalent metal ion has been shown to serve as a base for its activity (Ferrer et al. 2005). In addition, there are other amino acids residues in the substrate-binding pocket into which the flavonoid fits properly or forms hydrogen bonds with the hydroxyl groups of flavonoids. Figure 4a shows the substrate-binding pocket of IOMT. This diagram clearly shows that the His257 is vital to substrate binding and catalysis. The negative-charged Glu316 stabilizes the protonated histindine residue. Moreover, several hydrophobic amino acids such as Tyr127, Phe142, Phe150, and Phe164 form a hydrophobic pocket in which diadzein can stably bind. POMT7 also has a histidine–glutamate network in the flavonoid binding pocket, which is critical for catalysis. In addition, Glu117 forms a hydrogen bond with the 4′-hydroxyl group of quercetin (Fig. 4b).

Biological Application of FOMTs

Flavonoids are known as antioxidants and have been shown to display antiviral, antibacterial, anticancer, and anti-inflammatory activities (Harborne and Williams 2000). Biological activities of some of *O*-methylated flavonoids are listed in Table 2. *O*-Methylated flavonoids have been isolated from various plants and their biological activities have been evaluated. *O*-Methylation of flavonoids can alter and produce novel biological activities. For example, 7-*O*-methylquercetin (rhamnetin) inhibits the formation of β-amyloid (Kim et al. 2005c), while quercetin does not have this activity. Moreover, depending on the position of *O*-methylation, different biological activities are observed. For example, 3′-*O*-methylquercetin prevents epithelial cell damage (Bao and Lou 2006) while 3-*O*-methylquercetin contains antiviral activity (Castrillo and Carrasco 1987).

Table 2 Biological activities of some *O*-methylated flavonoids

O-methylated flavonoid	Common name	Biological activity	Reference
4′-*O*-methylapigenin	Acacetin	Inhibition of growth and induction of apoptosis in prostate cancer cell	Singh et al. 2005
3,4′-*O*-Dimethylkaempferol	Ermanin	Antiviral and antibacterial activity	Robin et al. 2001
3′,5′-*O*-Dimethyltricetin	Tricin	Inhibition of growth of colon cancer	Hudson et al. 2000
7-*O*-methyldaidzein	Formononetin	Estrogen-like activity	Jarry et al. 1985
3′-*O*-methyleriodictyol	Homoeriodictyol	Bitter taste making activity	Ley et al. 2008
3′-*O*-methylluteolin	Chrysoeriol	Anti-inflammatory activity	Williams et al. 1999
7-*O*-methylnaringenin	Sakuranetin	Inhibition of germination of *Magnaporthe grisea*	Kodama et al. 1992
4′-*O*-methylnaringenin	Isosakuranetin	Inhibition of *Helicobacter pylori* growth	Fukai et al. 2002
3′-*O*-methylquercetin	Isorhamnetin	Prevention of epithelial cell injury	Bao and Lou 2006
7-*O*-methylquercetin	Rhamnetin	Inhibition of β-amyloid formation	Kim et al. 2005c
3-*O*-methylquercetin	NA	Antiviral activity	Castrillo and Carrasco 1987

Therefore, regioselective *O*-methylation is an important factor to produce the specific *O*-methylated flavonoids with specific activities.

Extraction from plant and chemical synthesis of flavonoids may be a solution to increasing the supply *O*-methylated flavonoids. However, the plant extraction methods require a large amount of plant materials and multiple purification steps. In addition, there are several difficulties associated with the chemical synthesis of regioselective *O*-methylated flavonoids. Thus, an enzymatic reaction or biotransformation using microorganisms or particular cell lines, i.e., biological method, could be used for the mass production. However, identifying and cloning FOMT genes is a prerequisite for this approach. Enzymatic *O*-methylation reactions require the expensive cosubstrate SAM. Biotransformation using microorganisms circumvent this issue because endogenous SAM can be used.

Biotransformation of flavonoids using transgenic *Escherichia coli* is carried out as follows. After making the transformant with a specific gene in an expression vector, the gene is induced and then, the flavonoid is added to the culture. The flavonoid goes into the cell through unidentified channels of *E. coli* and undergoes a reaction. The reaction product is then exported into the culture medium. This approach has been successfully demonstrated with *E. coli* harboring various flavonoid biosynthetic genes (Willits et al. 2004). In order to produce an *O*-methylated flavonoid, *E. coli* harboring one *FOMT* was used (Kim et al. 2005a, b, 2006a, c). In addition, *O*-dimethylated flavonoid was also produced using *E. coli* harboring two *FOMT*s (Kim et al. 2005a, b). A combination of other flavonoid biosynthetic genes with FOMT was tried to produce an *O*-methylated flavonoid from a cheaper substrate (Kim et al. 2010b).

The productivity of *O*-methylflavonoid is important. Productivity of most *O*-methylflavonoids using biotransformation ranges from 20 to 60 mg/l. However, the productivity can be increased by adopting different approaches. First, the selection of the best FOMT is a prerequisite. Some FOMTs have the same substrate specificity but the kinetic parameters such as K_m and k_{cat} are different. It has been thought that enzymes containing better kinetic parameters are better for the biotransformation; however, in practice this is not always the case (Koffas 2010). Second, the copy number of the expressed gene is important. Plasmids maintaining a higher copy number in *E. coli* produce more proteins but also increase the metabolic load on the host. Thus, the selection of the best plasmid that maximizes the expression of FOMT and minimizes the metabolic load on the host is critical. Third, *O*-methylation requires the cofactor SAM as a methyl group donor. Engineering the SAM pathway to increase the production of SAM may increase the efficiency of product formation. Finally, after selection of the best FOMT, the expression vector and engineering of the SAM pathway, the optimal fermentation conditions must be determined. It has been estimated that the production of *O*-methylated flavonoid after optimization can be as high as 500–1,000 mg/l.

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